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OSMOTIC STRESS INDUCED ANTHOCYANIN PRODUCTION IN CELL SUSPENSION CULTURE OF POPLAR (*Populus deltoides* var. *occidentalis*)

©

BY

ASHOK THOLAKALABAVI

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

DEPARTMENT OF FOREST SCIENCE

EDMONTON, ALBERTA

FALL 1993

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Osmotic stress induced anthocyanin production in cell suspension culture of poplar (*Populus deltoides* (Marsh.) Bartr. var. *occidentalis* Rydb.)** submitted by **Ashok Tholakalabavi** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Forest Science (Tissue Culture).**

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Dedicated to

Seema, Akshay,

Appayya, Amma

and to all my family members

ABSTRACT

A cell suspension culture of poplar (*Populus deltoides* (Marsh.) Bartr. var *occidentalis* Rydb.), accumulating the anthocyanin pigment cyanidin 3-glucoside in the lag phase of culture growth, was subjected to osmotic stress with glucose and mannitol. Osmotic stress treatments resulted in growth suppression and higher anthocyanin accumulation compared with unstressed cells. Both an increase in the proportion of pigmented cells and an increase in the concentration of anthocyanin in the pigmented cells were responsible for high anthocyanin content of cultured cells subjected to osmotic stress. Osmotic stress induced by glucose suppressed growth more compared with that of mannitol and produced higher anthocyanin levels. Evidence is presented to show that not only the quantity of soluble sugars and amino acids, but also their ratio might influence growth and anthocyanin production of osmotically stressed cells.

The activities of phenylalanine ammonia lyase (PAL, EC 4.3.1.5) and tyrosine ammonia lyase (TAL, EC 4.3.1) and the role of accumulated organic solutes on PAL activity were determined. Both PAL and TAL activities were detected in the cells and they were found to be separate enzymes. The activities of both the enzymes increased in response to osmotic stress, indicating that both PAL and TAL play a regulatory role in anthocyanin biosynthesis of these cultured cells. However, PAL activity was upto 9 times higher than TAL activity. Increased PAL activity of osmotically stressed cells was associated with increased levels of PAL protein. The amount of phenylalanine present in the cells did not appear to play a role in influencing PAL activity, but both tyrosine and tryptophan influenced *in vitro* PAL activity. Among the phenolic acids, only shikimic acid accumulated in the cells in detectable levels. However, it did not influence *in vitro* PAL activity. Although none of the nonaromatic amino acids and soluble carbohydrates were found to influence *in vitro* PAL activity, the possible role of some of them in influencing *in vivo* PAL activity is discussed in view of their cellular content and their known effect on *in vivo* PAL activity.

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Date: June 27, 1993

Ashok Tholakalabavi

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Chapter I

GENERAL INTRODUCTION

Apart from the primary metabolites, plants produce certain compounds which do not have any clear function in the vital primary processes of plant cells. These secondary metabolites of plants such as alkaloids, essential oils, steroids and phenolics are used in pharmaceutical, food and cosmetic industries. Although some of these phytochemicals have been synthesized in laboratories, it has not been possible to synthesize certain phytochemicals having complex chemical structure.

Success in plant tissue culture is mainly due to the theoretical concept of cell culture imagined in 1902 by the German botanist Haberlandt (see Krikorian & Berquam for English translation). Plant cells in culture have been found to produce a wide range of secondary compounds (Parr 1989). The growth of cell suspension cultures as measured by fresh weight, dry weight, cell volume or cell number takes the form of a sigmoidal curve. Thus a period of little growth (lag phase), and a period of exponential growth followed by a period of steady growth (linear phase), which in turn is followed by a gradual cessation of growth as nutrients become depleted (stationary phase), occur in a cell suspension culture (Lindsey and Yeoman 1985). Most of the cell cultures accumulate secondary metabolites during the stationary phase (Mantel & Smith 1983). However, there are also a few reports on the production of secondary metabolites in the lag (Do & Cormier 1990) and log phase (Sakuta et al. 1986).

Rapid developments in the 1960's and 1970's have converted tissue and organ culture from an area primarily of academic interest into a major tool for plant propagation, crop improvement and synthesis of a variety of natural products such as secondary metabolites (Fowler 1983). Plant tissue culture has the potential to be an alternative means of producing the secondary metabolites provided secondary metabolite production by tissue culture is reliable and predictable. Also, compounds which are not synthesized in the plants are also sometimes synthesized by tissue cultures (Yeoman et al. 1980). It is also possible to convert low value precursors into high value products by biotransformation (Staba 1976). The products can be harvested in a short period of time and secondary compound extraction from tissue culture cells is simpler compared to their extraction from complex tissues of plants. By selecting cell lines producing only one secondary compound, the problem of separating the different secondary compounds present in an intact plant tissue can be avoided. As the pressure on land is increasing due to the growing population, production of secondary metabolites in tissue culture leaves more area for food production (Mantel et al. 1985).

Plant tissue culture offers an effective system for biosynthetic studies. Cultured cells offer many advantages over intact plants for such studies. Cultured cells can be grown under strictly controlled nutritional and environmental conditions and they provide simpler and more convenient experimental systems than intact plants (Butcher 1976).

Anthocyanins are a wide-spread group of plant pigments. They form a major group of secondary metabolites. These intensely colored water soluble pigments are responsible for nearly all the pink, scarlet, red, mauve, violet and blue colors in the petals and leaves of higher plants (Harborne 1967). Their functions in plants include, attraction of insects and animals for pollination and seed dispersal, providing protection from pathogens and UV light (Harborne 1967) and possibly serving as a reserve source of carbon (Gershenzon 1984).

Anthocyanins are used by man as food colorants. The safety of certain synthetic pigments has been in question, leading to progressive interest in the use of natural coloring compounds in food processing and manufacture (Francis 1984). Anthocyanins have been found to be produced in tissue cultures of several plant species (Seitz & Hinderer 1988) including poplar (Matsumoto et al. 1973).

Despite the advantages of plant tissue culture for the production of secondary compounds, its commercial application is of limited success (Thorpe 1990). Failure to produce the secondary metabolites found in the source plant, low level of production, and gradual decrease in product level upon subculturing are some of the reasons for this lack of success (Constabel 1987).

Not all cells in a culture produce secondary metabolites. Hall and Yeoman (1986) observed in *Catharanthus* cell cultures that even at maximum anthocyanin level, the proportion of productive cell population was only 10%. Also, considerable variation existed in the anthocyanin content of individual cells and they observed a large preponderance of cells containing low anthocyanin concentration. Therefore, there appears to be scope for increasing the production of secondary metabolites in cell cultures.

In the early days of plant tissue culture, culture media were developed to support rapid growth. Cultural conditions favoring rapid growth are seldom optimal for the biosynthesis of secondary metabolites (Luckner 1980). It has been possible however, to increase the accumulation of secondary metabolites by optimization of specific culture conditions (Mantel et al. 1985).

The accumulation of secondary metabolites in a cell culture is the result of a dynamic balance between the rate of its biosynthesis and the rate of its biodegradation. Therefore, culture conditions that promote biosynthesis of the desired compounds while limiting biodegradation are the best for secondary metabolite accumulation (Mantel et.al. 1985). Various culture conditions have been found to influence the production of secondary metabolites in plant tissue cultures. Osmotic stress is one such culture condition (DiCosmo & Towers 1984). Osmotic stress has been shown to increase anthocyanin production in cell suspension culture of grape (Do & Cormier 1990).

Knowledge of the enzymes which participate in the synthesis of secondary metabolites is essential for an understanding of secondary metabolism (Becker 1987). In general, there is a void in our knowledge of both enzymology and the genetic regulation of secondary product synthesis (Whitaker & Hashimato 1986). Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) is the first enzyme in general phenylpropanoid pathway and is considered to be a regulatory enzyme in the biosynthesis of several secondary metabolites. Although, in most cases PAL activity is correlated with secondary metabolite production (Becker 1987), there are also examples showing lack of correlation between the two (Constabel et al. 1971). Jones (1984) stated that there is considerable uncertainty concerning the regulation of PAL .

Amino acids (Nakamichi et al. 1983) and phenolic acids (Boudet et al. 1971; Alibert et al. 1972) have been shown to influence PAL activity. Sugars and amino acids are the major osmotic solutes accumulated in the cells in response to water stress. Organic acids also contribute to osmoregulation (Morgan 1984). The effect of solutes, accumulated in response to osmotic stress on PAL activity has not been investigated in cultures showing correlation between secondary metabolite production and PAL activity.

This dissertation presents the results of studies conducted using a cell suspension culture of poplar (*Populus deltoides* var. *occidentalis*). The research was conducted to understand the relationship between osmotic stress-induced increase in anthocyanin production and cell growth, solute composition and phenylalanine ammonia lyase activity.

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Chapter II

LITERATURE REVIEW

II.1 Introduction

In general, primary metabolites provide the basis for growth, while secondary metabolites provide the basis for interaction with and adaptation to the environment (Crocomo et al. 1981). The secondary metabolites of plants are being utilized by man as medicines, flavorings, tanning agents and food colorants. Anthocyanins, the intensely colored plant pigments, are one of the groups of secondary metabolites (Harborne 1967) which occur widely in gymnosperms, monocotyledons and dicotyledons. Anthocyanins are glycosides with polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts as aglycone. Sixteen different anthocyanidins are found in plants and a large number of glycosyl and acyl groups, which bind to these anthocyanidins, have contributed to more than 247 different anthocyanin pigments. The naturally occurring anthocyanins are glycosides of mainly six anthocyanidins, namely pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Jackman et al. 1987). The glycosides are water soluble and are stored in the vacuolar sap of mature cells. The most common anthocyanidin, cyanidin, exists in about 40 different glycosidic structures (Harborne 1980).

Various secondary metabolites, including anthocyanins, are produced in tissue cultures of several plant species. The advantage of commercial production of secondary metabolites by tissue cultures has been pointed out by several workers (Ganapathi & Kargi 1990; van derHeijden et al. 1989; Becker 1987; Staba 1982; Yeoman et al. 1980). Production of anthocyanins in tissue culture is helpful in the study of secondary metabolism. Various culture conditions are known to influence anthocyanin production in tissue cultures. Osmotic stress is one such culture condition (Do & Cormier 1990; DiCosmo & Towers 1984). The enzyme phenylalanine ammonia lyase (PAL, EC 4.3.1.5) is considered to be a regulatory enzyme of phenylpropanoid pathway. PAL activity is known to be induced under stress conditions. Amino acids and phenolic acids are also known to influence PAL activity (Hanson & Havir 1981). Various solutes are known to accumulate in water or osmotically stressed cells, of which soluble sugars, free amino acids and organic acids are the major organic solutes. This review covers the following aspects: anthocyanin pigments of poplar; production of secondary metabolites including anthocyanins in tissue culture, effect of osmotic stress on secondary metabolite production with particular reference to anthocyanins, relationship between PAL and TAL activity and secondary metabolite production, and regulation of PAL activity.

II.2 Anthocyanins of poplar

Anthocyanins are responsible for the bright coloration of young leaves and catkins of poplar. All the anthocyanins of poplar are found to be glycosides of cyanidin. Bendz & Haglund (1968) identified the anthocyanins present in the young leaves and catkins of *Populus tremula* as cyanidin 3-glucoside, cyanidin 3-xylosylglucoside and cyanidin 3-rhamnosylglucoside. They also found the same pigments in a hybrid poplar (*Populus alba* x *Populus tremula*). Chang et al. (1989) reported the presence of cyanidin 3-glucoside and cyanidin 3-galactoside in the leaves of *Populus tremuloides*. Anthocyanins have also been found to be present in the roots of poplar (Ketshoveli 1978).

There is only one report on anthocyanin production in tissue culture of poplar. Matsumoto et al. (1973) identified the anthocyanin produced in cell suspension culture of a hybrid poplar (*Populus nigra* x *Populus maximiwiczii*) as cyanidin 3-glucoside.

II.3 Production of secondary metabolites in tissue culture

De novo biosynthesis of secondary metabolites *in vitro* has been well established (Kurz & Constabei 1979). There are several reports on the production of various groups of secondary metabolites such as alkaloids (van der Heijden 1989), steroids (Reinhard &

Alfermann 1980), essential oils (Mulder-Krieger et al. 1988), phenolics (Parr 1989) and betalains (Berlin et al. 1986) in plant tissue culture. Generally, the compounds that are produced in many kinds of tissue culture are those that 1) are wide spread in plants such as phenolics, including anthocyanins, 2) occur in plant cells which do not feature special structures, or 3) are biologically less complex (Constabel 1987).

The use of cell and tissue cultures in the studies of secondary metabolism has achieved significance during the 1970s and 1980s. Much of this work is related to the potential for producing commercial products (Thorpe 1990). Plant tissue culture could be used as an alternative to whole plants as a biological source of secondary metabolites of high economic value. Use of cell cultures to produce secondary metabolites offers certain advantages over the conventional cultivation of plants. These are, 1) compounds could be produced under controlled conditions, ensuring a steady supply, 2) metabolic processes could be regulated to increase the yields, as cell cultures are simpler systems than whole plants, 3) cells could be genetically modified to accumulate specific intermediates and metabolites (Kurz & Constabel 1979), and 4) certain cell cultures might produce novel secondary metabolites which are not produced by the plant (Ibrahim 1987).

To be useful as an alternative industrial source of secondary metabolites, a cell culture must satisfy several requirements. 1) A good yield of final product is essential. 2) The cells must be genetically stable and should maintain the high yielding capacity over several subcultures to give a constant yield of products. 3) The production must be profitable. This would limit the commercial scale production by tissue culture to secondary metabolites of high economic value (Crocomo et al. 1981).

Cell cultures have also served as an efficient system for the study of biosynthesis, biotransformation and regulation of metabolite synthesis. The isolation of cells from cellular interaction, which is a characteristic of cells in plant tissues, makes them simpler systems for the study of secondary metabolism at the cellular level. Strict control of environmental conditions, direct exposure to chemical and physical factors and various cell manipulations contribute to the preferential use of cell cultures for studying the synthesis and accumulation of phytochemicals. However, whether insights obtained by using cell cultures reflect the events occurring in the plant needs to be assessed, if the aim of the study is to understand the mechanisms occurring in the plant (Constabel 1987). The findings of these studies can also be used to enhance the production of secondary metabolites, to make their production by cell culture a commercial feasibility.

The most important prerequisite for the use of plant cells in the commercial production of secondary metabolites is their ability to grow in liquid media, which is referred to as cell suspension culture. Of the different methods of cell suspension cultures employed, batch culture is the most widely used. In this system the cells are grown in a fixed amount of nutrient, forming a population of cells in which succeeding generations progressively modify their environment. Batch culture method has its place in the production of biomass and for exploratory research on the biosynthesis of secondary metabolites (Kurz & Constabel 1979). In general, most secondary metabolites accumulate during the later rather than early stages of growth of batch cultures. During the lag phase of culture growth, new amino acids and proteins are synthesized, during the exponential phase biomass increases and during the stationary phase major metabolic events leading to secondary product formation occur (Staba 1982). However, in certain cell cultures secondary metabolites can accumulate rapidly even during the log (Mantel et al. 1985) or lag (Do & Cormier 1990) phase of growth. The amount of a secondary metabolite at any particular instant in the growth of a culture is a result of the dynamic balance between the rate of its synthesis and the rate of its degradation. It has been shown in several cell cultures that there is a turnover and degradation of secondary metabolites (Thorpe 1990).

Lack of production of secondary metabolites found in the source plants, low level of production, and gradual decrease in product formation upon subculturing are some of the problems encountered in the production of secondary metabolites by tissue culture. By 1987, there were thirty cell culture systems that were better producers of secondary metabolites than the respective plants (Wink 1987). Unfortunately many of the economically important plant products are either not produced or not produced in sufficiently large quantities by plant cell cultures (Thorpe 1990). Constabel (1987) observed that the cause for this lack of accumulation of secondary metabolites found in the source plant could be due to the following reasons. 1) Lack of concomitant structural features 2) Lack of precursors 3) Absence of enzyme activity and 4) Degradation of products. Bohm (1982) reviewed the causes for the inability of plant tissue cultures to produce secondary metabolites, and concluded that lack of specific enzymes represents the most important reason.

Although the secondary metabolites which accumulate in special compartments are generally not expected to occur in callus or cell cultures, some terpenoids and alkaloids restricted to special cells in plants have been observed to be produced in cell cultures (Mulder-Krieger et al. 1988; Constabel 1987). Although certain cell cultures have been observed to retain the capacity for the production of the secondary metabolites over many subcultures (Dougall 1979), in several instances yields of secondary metabolites gradually declined with time (Crocomo et al. 1981). However, the plants regenerated from the cell cultures recovered the patterns and levels of alkaloids characteristic of the source plant (Dougall 1979). These evidences indicate that secondary metabolite production is linked to morphological differentiation. This finding has stimulated interest in the use of organ cultures (Thorpe 1990). A number of hairy root systems have been successfully used to enhance the production of secondary metabolites *in vitro* (Kamada et al. 1986).

Inverse relationship between growth and secondary metabolite production (Sakuta & Komamine 1987) and cellular heterogeneity (Hall & Yeoman 1986) has been well established in many cell cultures. The high secondary metabolite accumulating cells might multiply slowly, whereas fast growing cells are nonproductive. This might result in a predominance of low secondary metabolite producing cells over several subcultures, resulting in low yields. Frequent selection of high secondary metabolite accumulating cells (cell line selection) and their multiplication has been shown to overcome this problem (Widholm 1987). A two stage culture, one for biomass production and the other for secondary metabolite production is adopted in certain cultures (Beiderbeck & Knoop 1987). Structural and biochemical heterogeneity exists at every stage of development of a cell culture (Lindsey & Yeoman 1985). Cell suspension cultures are genetically heterogeneous with single cells and cell aggregates growing at different rates (Kurz & Constabel 1979). The range of chromosome numbers in the cultured cells of *Datura innoxia* was wider than the range in the plants regenerated from the cultures (Hiraoka & Tabata 1974). This will also contribute to the heterogeneity of cultured cells. A well growing suspension will consist of at least 50% cells which are about to divide, a number of quiescent cells and also senescent cells (Chu & Lark 1976). The actively growing cells in a culture are likely to accumulate low levels of secondary metabolites. A proportion of cell population might enter the stationary phase early and then shifts to secondary metabolism (Kurz & Constabel 1979). All these factors might contribute to the heterogeneity of cell cultures with respect to secondary metabolite production.

A better understanding of the cause for the cellular heterogeneity might be helpful in inducing the production of secondary metabolites in a larger proportion of cells in a culture. Commercial production of shikonin (Fujita & Tabata 1987), and attempts to commercialize the production of berberine (Kobayashi et al. 1988), rosmarinic acid (Ulbrich et al. 1985), taxol, sanguinarine and ginseng (Payne et al. 1991) by cell and tissue culture methods are very encouraging. Continued basic and applied research in this area should enable enhanced production of secondary metabolites, and commercialization of the production of many high cost secondary metabolites might be possible in the near future.

II.3.1 Production of anthocyanins in tissue culture

Anthocyanins are found to be produced in callus and cell suspension cultures of 27 different plant species. In general, it can be assumed that plant cells cultivated *in vitro* retain the capacity for producing the same anthocyanins as *in vivo* (Seitz & Hinderer 1988). In a callus, pigment containing cells may be distributed diffusely or localized, giving a patchy appearance to the cultures (Butcher 1976). The anthocyanins produced in tissue cultures of *Machaeranthera gracilis* (=*Haplopappus gracilis*) (Stickland & Sunderland 1972; Constabel et al. 1971; Reinert et al. 1964) and *Daucus carota* (Ozeki & Komamine 1982) have been extensively studied.

Ardenne (1965) identified the anthocyanins present in a highly pigmented strain of *Machaeranthera gracilis* as cyanidin 3-glucoside and cyanidin 3, 5-diglucoside. Stickland & Sunderland (1972) found cyanidin 3-glucoside and cyanidin 3-rutinoside in another strain of *Machaeranthera gracilis*. Glycosides of cyanidin, delphinidin, malvidin, petunidin, pelargonidin, peonidin and hirsutidin have been isolated from callus cultures of different plant species.

Not all cells in a culture produce anthocyanins. Hall & Yeoman (1986) observed in *Catharanthus* cell cultures that even at maximum anthocyanin level the proportion of productive cell population was only 10%. Also, considerable variation existed in the anthocyanin content of individual cells. They observed a large preponderance of cells containing low anthocyanin concentration.

II.4 Effect of osmotic stress on growth and secondary metabolite production

Several culture conditions influence the production of secondary metabolites by plant tissue cultures. Culture conditions favoring rapid growth are generally not suited for the biosynthesis of secondary metabolites (Luckner 1980). Osmotic stress is one of the culture condition influencing secondary metabolite production in tissue cultures.

It is a common observation that increased sucrose concentrations in tissue culture medium improve secondary metabolite yields (van der Heijden et al. 1989). An increase in sugar concentration from the 1-2% level commonly used in the growth medium to between 5-10% brings about enhanced secondary metabolite yields (Morris et al. 1985).

Anthocyanin production in callus culture of a hybrid poplar increased with the increase in the concentration of sucrose from 0.3% to 5%, while growth was not affected (Matsumoto et al. 1973). However, Do & Cormier (1991) observed an increase in anthocyanin production by an increase of medium sucrose concentration in grape cell suspension culture, but cell growth was inhibited. Knobloch et al. (1982) found anthocyanin and alkaloid synthesis in *Catharanthus roseus* cell suspensions to increase when sucrose concentration was increased from 4% to 10%. Higher anthocyanin accumulation in carrot cell suspension culture was observed when sucrose level in the medium was increased from 2% to 9% (Ozeki & Komamine 1985). However, high sucrose concentrations led to inhibition of anthocyanin production in certain other cell cultures of *Dimorphotheca sinuata* and *Petunia hybrida* (Seitz & Hinderer 1988).

Knobloch & Berlin (1980) were able to stimulate alkaloid production by transferring *Catharanthus roseus* cells to a medium containing 8% sucrose. Increasing the sucrose concentration from 1% to 5% increased the shikonin yield in *Lithospermum erythrorhizon* callus cultures (Mizukami et al. 1977). Davies (1972) observed a stimulation of polyphenol synthesis in cell suspension cultures of rose when the medium sucrose concentration was increased from 2% to 4%. A three-fold increase in phenolic metabolite accumulation in cell suspensions of *Acer pseudoplatanus* was observed when sucrose concentration was increased from 2% to 4%. (Westcott and Henshaw 1976). Constabel (1968) found 5% sucrose to enhance tannin production in tissue culture of *Juniperus communis*.

Increase in secondary metabolite production induced by an increase in the concentration of sugar may be due to both a relief of carbon limitation and due to the stress caused by the decreased osmotic potential of the medium (Collinge 1986). Decreasing the osmotic potential of the medium by the addition of osmotica such as mannitol or polyethylene glycol has been found to increase secondary metabolite production in tissue cultures. Anthocyanin content of grape cell suspension culture was enhanced by low osmotic potential of the medium induced by the addition of mannitol to the medium (Do & Cormier 1990 & 1991). Sakuta et al. (1987) however, found a decrease in betacyanin production in *Phytolacca americana* with the decrease in medium osmotic potential brought about by the addition of mannitol to the culture medium, at a constant sucrose concentration. Morris et al. (1986) observed that mannitol reduced cell growth in *Catharanthus* cell cultures based on cell number and cell size. Water stress could lead to increased levels of phenolic compounds by reducing the rate of growth leading to a build up of precursors (Gershenzon 1984). Control on secondary product formation induced by osmotic stress treatment might lead to a better understanding of the cytodifferentiation process.

II.5 Arylalanine ammonia lyase activity and secondary metabolite production

An essential condition for an understanding of secondary metabolism is to know the enzymes which participate in the synthesis of these substances (Becker 1987). Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) is the first committed enzyme of the phenylpropanoid metabolism in higher plants which catalyzes the elimination of ammonia from L-phenylalanine to give *trans* cinnamic acid. The enzyme derives its importance in the biosynthesis of several secondary metabolites by being the first enzyme involved in the diversion of phenylalanine from primary metabolism to secondary metabolism. The enzyme from many sources, especially from monocotyledons acts also on L-tyrosine to yield *trans p*-coumaric acid and therefore, is said to show tyrosine ammonia lyase (TAL, EC 4.3.1) activity (Hanson & Havir 1981). In fact, it has been shown for the maize enzyme that both PAL and TAL activities occur at the same catalytic site (Reid et al. 1972). However, there are also reports which show that PAL and TAL are two different enzymes (Beaudoin-Eagan & Thorpe 1985; Gregor 1976; Kindl 1970). Although TAL activity was detected mostly in monocotyledons, there are also a few reports on the presence of TAL activity in a few dicotyledonous species. Beaudoin-Eagan & Thorpe (1985) found both PAL and TAL activity in tobacco callus culture and Nagaoka & Ashihara (1988) found the activities of both the enzymes in *Catharanthus roseus* cell suspension cultures. Gregor (1976) found both PAL and TAL activities in castor bean endosperm. TAL activity was also observed in soybean (Chiang et al. 1987). PAL activity was almost always higher than TAL activity (Nagaoka & Ashihara 1988; Hanson & Havir 1981; Green et al. 1975), and therefore PAL might play a more important role than TAL in the biosynthesis of secondary metabolites. Siriphanich & Kader (1985) could not detect TAL activity in lettuce.

PAL is considered to act as a regulatory enzyme in the biosynthesis of several secondary metabolites including anthocyanins. Higher sucrose level in the medium brought about a marked increase of PAL activity during the lag phase, preceding anthocyanin production in the callus of a hybrid poplar (Matsumoto et al. 1973). The anthocyanin-containing cell line of carrot was found to have higher PAL activity and showed higher *de novo* synthesis of PAL compared with the anthocyanin-free cell line (Heinzmann & Seitz 1977). In the cotyledons of mustard seedlings, appearance of PAL activity was a prerequisite for the production of anthocyanins (Brodenfeldt & Mohr 1988). Inhibition of PAL activity by L-2-aminooxy-3-phenylpropionic acid (AOPP) in buckwheat hypocotyls also resulted in the inhibition of anthocyanin formation (Amrhein & Godeke 1977; Amrhein et al. 1976). In *Petroselinum hortense* cell cultures, the amount of flavonoids accumulated was correlated with PAL activity but not with any other enzymes involved in the flavonoid pathway (Hahlbrock et al. 1980). In *Catharanthus roseus* cell suspension cultures, increase of both PAL and TAL activities were found to coincide with the increase in the level of phenolic acids (Nagaoka & Ashihara 1988). Similar correlation between PAL and TAL activities, and phenolic content of soybean leaves were observed (Chiang et al. 1987). Guerra et al. (1985) reported a correlation between PAL and TAL activities, and lignin synthesis in wheat. Contrary to these observations, Constabel et al.

(1971) found no correlation between PAL activity and the rate of anthocyanin accumulation in *Haplopappus gracilis* cell suspension cultures. Weissenbock (1975) observed that PAL and TAL are not the rate limiting enzymes in the biosynthesis of flavones in oat seedlings.

II.5.1 Regulation of phenylalanine ammonia lyase and tyrosine ammonia lyase activity

The classic work of Hahlbrock et al. (1980) and Ebel & Hahlbrock (1977) showed that the enzymes of the general phenylpropanoid pathway (group I enzymes) and of the flavonoid glycoside pathway (group II enzymes) were coordinatedly induced in parsley and soybean cell cultures. They concluded that phenolic synthesis in cultured cells is regulated at the enzyme level by the high substrate specificities of the individual enzymes of the above pathways, the existence of isozymes and the coordinated changes in enzyme activities by various exogenous and endogenous factors. Considering the reports on the regulation of enzyme activities responsible for the flavonoid biosynthesis, Seitz & Hinderer (1988) concluded that phenylalanine ammonia lyase (PAL), which leads to the general phenylpropanoid pathway, and chalcone synthase, which leads to the flavonoid glycoside pathway, both located at the branching points of the respective pathway, are the key enzymes in the biosynthesis of phenolic compounds.

Jones (1984) stated that to explain the magnitude and timing of PAL induction there must be changes in the number of catalytically competent enzyme molecules rather than large variation in the catalytic efficiency of a fixed amount of the already existing enzyme. Increased *de novo* synthesis of PAL has been observed when an increase in PAL activity occurred (Wellman & Schopfer 1975; Nakamichi et al. 1983). Increase in the activity of a pre-existing pool of inactive PAL also might contribute to its increased activity (Lau et al. 1980; Jones 1984). In addition to the production of PAL activity, its removal by inactivation or degradation must be postulated to explain the decline in PAL activity observed with time in many tissues (Jones 1984). The loss of PAL activity observed in many tissues may be attributed to the presence of the PAL inactivating system. Zucker (1971) reported that control of PAL activity in cocklebur leaves was primarily through control of the level of the PAL inactivating system. PAL activity was found to increase in excised hypocotyl tissue of sunflower, incubated in sucrose solution. Experimental evidences indicated that *de novo* synthesis of PAL, and subsequent synthesis of a PAL-inactivating system were responsible for the increase of PAL activity and subsequent decrease in its activity (Tena et al. 1984). Various mechanisms involved in the appearance and disappearance of PAL activity in different plant species have been extensively reviewed by Hanson & Havir (1981) and Jones (1984).

Short term interactions between metabolites and enzymes might provide a fine tuning of the metabolic pathway. Various amino acids, when supplied through the medium have been shown to increase PAL activity in the yeast *Rhodotorula glutinis* by increasing its *de novo* synthesis. Isoleucine was found to contribute to the stability of PAL activity by inhibiting the synthesis of the PAL inactivating system (Nakamichi et al. 1983). PAL activity has been shown to be regulated by the supply of its substrate phenylalanine in *Phaseolus vulgaris* (Cunha 1987). There must be interactions between the metabolites and enzymes, especially the intermediary metabolites of the shikimic acid, phenylpropanoid and flavanoid pathway on the activity of the regulatory enzymes of secondary metabolism (Hanson & Havir 1981). Gallic acid was found to stimulate PAL activity, while benzoic, caffeic and ferulic acids inhibited PAL activity (Boudet et al. 1971; Alibert et al. 1972). Homogentisic acid reduced the activity of PAL from barley (Kindl 1970). *para*-Coumaric acid and *trans*-cinnamic acid were found to inhibit the activity of PAL from barley (Koukol & Conn 1961).

Osmotic stress results in the accumulation of various solutes. Sugars and amino acids are the major osmoregulatory solutes in many plant species (Zwiazek & Blake 1990; Fallon & Phillips 1988; Jacomini et al. 1988). Plants also accumulate organic acids in response to osmotic stress (Rudge & Morris 1987). These accumulated solutes might influence enzyme activities.

PAL is a tetramer with only two active sites. It is known that enzymes undergo major conformational adjustments on ligand binding. These changes may include interactions among protomers so that binding on one protomer influences binding at a similar site on another protomer (Hanson & Havir 1981). Different ligands may move the enzymes to binding states that are the same or distinct.

In contrast to the literature available on the regulation of PAL, little is known about the regulation of TAL. In fact, TAL was found to be a separate enzyme in only a few cases (Beaudoin-Eagan & Thorpe 1985; Gregor 1976; Kindl 1970). In castor bean, where TAL was shown to be a separate enzyme, wounding of seedlings caused a strong increase in PAL activity, but did not affect TAL activity. Treatment of seedlings with manganese chloride drastically enhanced PAL activity, but only a slight increase of TAL activity was observed (Gregor 1976). Kindl (1970) studied the effect of various phenolic compounds on *in vitro* activity of TAL from barley, which was found to be a different enzyme. They concluded that a 2,5-hydroxyphenyl structure was the most effective inhibitor of TAL activity, and aromatic compounds with a 2-hydroxy group have no or little influence. In addition to the metabolites of L-tyrosine, p-coumaric acid and p-hydroxybenzoic acid, homogentisic acid, a product of the catabolism of L-tyrosine leading to aliphatic dicarboxylic acids, were also effective inhibitors of TAL activity.

In summary, the review of literature showed that PAL activity and secondary metabolite production increase under osmotic stress conditions, that sugars, amino acids and organic acids accumulate in the cells in response to osmotic stress, and that certain amino acids and phenolic acids influence PAL activity. However, there have been no studies conducted to link all these three aspects together. Therefore, there is a need to learn more about how solutes accumulated in the cells in response to osmotic stress affect

PAL activity in those cell cultures which show a correlation between secondary metabolite production and PAL activity. Such studies would give a better understanding of the relationship between primary and secondary metabolism. Knowledge of the mechanisms involved in increased secondary metabolite production by osmotic stress will enable a more focused approach to be adopted in the manipulation of plant tissue and cell cultures to produce higher product yields.

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CHAPTER III

Effect of mannitol and glucose induced osmotic stress on growth, water relations and solute composition of cell suspension culture of poplar (*Populus deltoides* var. *occidentalis*) in relation to anthocyanin accumulation

III.1 Introduction

Plant cell cultures that produce secondary metabolites serve not only as potential alternative systems for the production of valuable compounds but also as useful systems for the study of secondary metabolism. The production of secondary metabolites in cell cultures is influenced by various culture conditions including osmotic stress (Mantel & Smith 1983). A high concentration of sugars in the medium is known to increase secondary metabolite production in cell cultures (van der Heijden et al. 1989). This effect of high sugar concentration in the medium could be either due to the osmotic effect or due to the role of sugars as a carbon source. To separate the osmotic effect of sugars from their role as a carbon source, investigators have substituted part of the sugars by mannitol and the resultant effect on growth and secondary metabolism has been attributed to the osmotic effect (Do & Cormier 1990). Although mannitol is not metabolized by many plant species, it might be taken up by certain cultured cells and metabolized, thus serving as a carbon source (Thompson et al. 1986). Also, because mannitol is considered as a compatible solute (Borowitzka 1981), it might influence cell growth and hence secondary metabolism in a different way than sugars when accumulated in the cells. Borowitzka (1981) explains compatible solutes as the low molecular weight, neutral, organic solutes capable of being accumulated to high concentrations without severe inhibition of cell processes. Although high concentrations of both sugars and mannitol in the medium have been shown to increase the yields of secondary metabolites in separate studies, a direct comparison of their osmotic effects has not been made.

Plant cells in batch culture are generally found to accumulate the maximum amount of secondary metabolites during the stationary phase of culture growth (Mantel & Smith 1983). However, there are a few reports of cell cultures accumulating secondary metabolites in the lag phase (Noguchi & Sankawa 1982) and even fewer reports on the effect of osmotic stress on secondary metabolite production in the lag phase (Do & Cormier 1990). At the lag phase of culture growth the supply of carbon will not be limited. Therefore, a cell culture accumulating maximum anthocyanin during the lag phase of culture growth allows us to compare the osmotic effects of two solutes differing in the degree to which these solutes are metabolized, under unlimiting conditions of carbon source.

The mechanism of osmotic stress induced secondary metabolite production in cell cultures producing them during the stationary phase and during the lag phase of culture growth might vary because the composition solutes in the cells changes greatly from lag to stationary phase (Lindsey & Yeoman 1985). However, there are no reports on the soluble carbohydrate composition of the cells osmotically stressed with sugars or sugar alcohols in relation to secondary metabolite production. Such information is necessary to understand the role of these solutes in secondary metabolite production, as these solutes might influence secondary metabolite production by influencing enzyme activity, substrate supply, accumulation process or degradation.

Cellular heterogeneity is a common feature of plant cell cultures (Hall & Yeoman 1986) and only a small proportion of cells in a culture accumulates secondary metabolites. An increased yield of secondary metabolites in a cell culture can be brought about by an increase in the proportion of productive cell population or by an increase in its concentration in the productive cells. In this study, changes in both the proportion of anthocyanin accumulating cells and the concentration of anthocyanin in the cells, due to osmotic stress induced by glucose and mannitol have been examined. It is hoped that this study will help in understanding the mechanism of osmotic stress-induced secondary metabolite accumulation at the cellular level.

An inverse relationship between growth and secondary metabolite accumulation appears to be a common feature of plant cell cultures (Mantel & Smith 1983). The objective of this study was to examine the effects of osmotic stress induced by two solutes, mannitol and glucose, on growth, water relations and solute composition in relation to anthocyanin production in a cell suspension culture of poplar (*Populus deltoides* var. *occidentalis*), which was found to produce anthocyanin in the lag phase of culture growth.

III.2 Materials and methods

III.2.1 Culture initiation and maintenance

Leaves of *Populus deltoides* (Marsh.) Bartr. var. *occidentalis* Rydb. were obtained from the Devonian Botanical Gardens, Devon, Alberta. Sterile leaf explants, about 0.5 cm ^{2} in size were used to initiate callus on Murashige and Skoog (MS) agar medium (1962) containing 3% sucrose and 5 µM each of 2,4-Dichlorophenoxyacetic acid (2,4-D) and kinetin. Those calluses which developed red pigments on their outer surfaces when exposed to light, were transferred to 250 ml flasks containing 100 ml MS liquid medium. The MS liquid medium contained 3% sucrose and 2uM 2,4-D and 1 µM kinetin, the growth regulator concentrations that were found to be optimal for anthocyanin production in a hybrid poplar cell suspension culture (Matsumoto et al. 1971). The cultures were kept agitated in a rotary shaker at 120 rpm. Cultures were maintained at room temperature (22°C under continuous wide spectrum fluorescent light (Gro and Sho, Model F40/GS/W9) at a light intensity of 40 µmol m^{-2} sec^{-1}. The soft red-pigmented part of the callus formed a suspension of small aggregates while hard and green inner portions were removed after 10 days. Cultures were transferred to fresh medium once in 10-15 days.

III.2.2 Anthocyanin identification

III. 2.2.1 Extraction of anthocyanin

The anthocyanin was extracted from about 20 g fresh weight of cells in 25 ml of 1% HCl in methanol. The extract was filtered and concentrated *in vacuo* at 30°C for use in paper chromatography. For spectral studies, the extract was washed with ethyl acetate and the aqueous fraction was concentrated as above. The concentrate was further purified by preparative paper chromatography using butanol-acetic acid-water (BAW) as the solvent system (Harborne 1984).

III.2.2.2 Paper chromatography of aglycone, sugar and anthocyanin

The anthocyanidin and the sugar were obtained by acid hydrolysis of anthocyanin (Harborne 1984). This was achieved by heating the anthocyanin in 2 M HCl for 30-40 min at 100°C. Standards of cyanidin, delphinidin and pelargonidin were obtained from rose petals, outer pericarp of egg plant fruit and pomegranate fruit respectively. Descending paper chromatograms were developed in Forestal (conc. HCl-acetic acid-water), Formic acid (conc. HCl-formic acid-water) and BAW (butanol-acetic acid-water) solvent systems.

The sugar component of the pigment was identified according to the procedure outlined by Markham (1982). The chromatogram was developed in descending mode in BAW, BEW (butanol-ethanol-water) and BBPW (butanol-benzene-pyridine-water) solvent systems, along with the standard sugars. The chromatogram in BBPW solvent system was over run to separate glucose and galactose. The dried chromatograms were sprayed with aniline hydrogen phthalate reagent and heated in an oven at 100-120°C for about 3 min to render the spots visible.

The number of sugar molecules was identified by controlled hydrolysis and the position of the sugar molecule by peroxide hydrolysis (Francis 1982). The anthocyanin was identified according to the procedure given by Harborne (1984). Standards of cyanidin 3-glucoside and cyanidin 3,5-diglucoside were obtained from rose petals. Poplar cell culture anthocyanin along with standard cyanidin 3-glucoside and cyanidin 3,5-diglucoside were run in BAW, BuHCl (butanol-hydrochloric acid) and 1% HCl solvent systems, in descending mode.

III.2.3 Water potential and osmotic potential determinations

The osmotic potential of the medium, and water and osmotic potential of the cells were determined using a HR-33T Wescor microvoltmeter equipped with a triple psychrometer chamber (Dixon Instruments Inc., Guelph, Ontario), in the dew point mode. Cell water potential was determined after filtering and spin drying the cells at 200x *g* for 5 min to remove surface moisture (Fallon & Phillips 1988). For the determination of the osmotic potential, the cells, wrapped in aluminium foil were frozen overnight at -30^0 C, followed by thawing. Samples were left in the sealed chamber for 90 min for cell water potential determination and for 30 min for cell and medium osmotic potential determinations. Preliminary experiments showed that this was sufficient time for equilibration of water content of the samples with that of the air in the psychrometer chamber. For dew point measurement, a 10-second cooling period was used and reading was taken 30-45 seconds after switching to the dew point mode. The microvoltmeter was calibrated against sodium chloride standards at room temperature (22^0 C).

III.2.4 Imposition of osmotic stress treatments.

A standard curve of glucose concentration in MS medium versus osmotic potential was obtained by experimental determination of osmotic potentials. An osmotic potential of -0.6 MPa was chosen as control (105 mM glucose), and -1.1 MPa (326 mM glucose) and -1.6 MPa (547 mM glucose) were chosen for glucose stress treatments. A standard curve of mannitol concentration versus osmotic potential in the control medium was obtained and an osmotic potential of -1.1 MPa (210 mM mannitol) and -1.6 MPa (450 mM mannitol) were chosen for mannitol stress treatments. Each treatment consisted of three replications.

Cells from 12-15 day-old cultures (linear phase of culture growth), at an inoculum density of 6.5% packed cell volume (PCV), were used in the experiment. Aliquots of cell suspension were taken every three days from the time of culture inoculation for up to twelve days. Packed cell volume (PCV), cell number, osmotic potential of media, and water potential, osmotic potential, anthocyanin, total soluble sugar and amino acid content of the cells were determined. The sugar composition of the cells was determined 12 days after culture inoculation.

III.2.4.1 Osmotic stress with polyethylene glycol (PEG)

The experimental conditions were the same as described earlier. PEG (MW 3350), at concentrations of 0, 30, 60 and 75 mM, was added to the MS medium containing 105 mM glucose, to get medium osmotic potentials of -0.6 MPa, -0.75 MPa, -1.25 MPa and -1.5 MPa. Cell dry weight and anthocyanin content were determined as described in the following sections.

III.2.5 Growth measurements

Packed cell volume (PCV) was determined by centrifuging the aliquots of culture at 200x *g* for 5 min (Dixon 1985). Percent packed cell volume (%PCV) was determined by calculating the PCV per 100 ml of culture. Cell dry weight was determined after filtering, washing and drying the cells for 12 h at 60^0C (Dixon 1985). Cell number and number of pigmented cells were determined only for control and -1.6 MPa stress levels of glucose and mannitol by taking aliquots of fine cell suspension and counting the pigmented and nonpigmented cells using a haemocytometer in a light microscope. The percentage of pigmented cells was calculated from the number of pigmented cells and the total cell number in 1 ml of culture. Amount of anthocyanin per packed pigmented cell volume was used as an indicator of anthocyanin concentration in the cells, and was calculated by dividing anthocyanin per ml of culture by packed pigmented cell volume per ml of culture. The packed pigmented cell volume was calculated from packed cell volume per ml culture and pigmented cell number per ml culture. Anthocyanin per pigmented cell was calculated by dividing anthocyanin per ml culture volume by the number of pigmented cells in 1 ml of culture.

III.2.6 Quantification of anthocyanin content of cells

The anthocyanin was extracted from the cells in methanol containing 1% HCl and the absorbance of the anthocyanin extract was measured at 525 nm. Because the anthocyanin pigment in this cell culture was identified to be cyanidin 3-glucoside, the molar extinction coefficient of this pigment was used to calculate the anthocyanin content (Francis 1982).

III.2.7 Determination of soluble sugars and free amino acids

The harvested cells were washed in deionized water and freeze-dried. Five mg of freeze-dried cells were ground in a glass mortar. Soluble sugars and free amino acids were extracted in 5 ml hot 80% ethanol for 30 min. Extracts were filtered and the residue was extracted twice in 80% ethanol. Filtrates were combined and evaporated to dryness *in vacuo* at 40^0C. Total soluble sugars were quantified as β-D-Glucose equivalents by the anthrone method (Ashwell 1957). The total free amino acids were quantified as L-leucine equivalents by the modified ninhydrin method (Rosen 1957).

For the determination of the soluble carbohydrate composition, extracts from 10 mg freeze dried cells were prepared as above. The dried carbohydrate extracts were dissolved in deionized water and passed through a 0.5 x 7.0 cm Dowex 50W (200 mesh) cation exchange column (H $^+$ form). The column was washed with 10 column volumes of deionized water and the eluate was applied to a 0.5 x 7.0 cm Dowex 50W (200 mesh) anion exchange column (Cl^- form). The anion column was washed with 10 column volumes of deionized water and the eluate was evaporated to dryness *in vacuo* at 40^0C. Trimethylsilyl (TMS) derivatives of carbohydrates were prepared as described by Zwiazek & Shay (1988). After overnight derivatization, TMS ethers of carbohydrates were analyzed using a Hewlett Packard 5890 gas chromatograph equipped with a Hewlett Packard 3396A electronic integrator. Samples were separated on a 30-m-long, 250 µm internal diameter J & W fused silica DB-5 capillary column, 0.25 µM film thickness. Conditions during the run were as follows: Initial oven temperature 180^0C, increased to 210^0C at a rate of 2^0C min^{-1}; followed by 210 to 250^0C at a rate of 5^0C min^{-1} and held for 10 min at 250^0C; injector and Flame Ionization Detector (FID) temperature 300^0C; 50:1 split injection; carrier gas, (helium) linear flow rate 25 cm sec^{-1}. Carbohydrates were identified by co-chromatography with carbohydrate standards and were quantified using an internal standard (sorbitol), which was added to all the extracts. Preliminary experiments showed that the cells did not contain sorbitol.

III.2.8 Determination of mannitol metabolism

Aliquots from 12 day-old cultures of -1.1 MPa and -1.6 MPa mannitol stress treatments were transferred to a 25 ml Erlenmeyer flask under aseptic conditions. (U-^{14}C) Mannitol was added to the culture to obtain a final specific activity of 20 Bq µmole^{-1}. A filter paper saturated with 25% KOH serving as a carbon dioxide trap was hung from the top of each flask, the flasks were sealed with a serum cap and incubated for 14 h in an orbital shaker at 120 rpm. At the end of incubation, the filter paper was placed in a scintillation vial containing the scintillation cocktail (Scintiverse E). The radioactivity was measured using a Minaxi β Tricarb 4000 series liquid scintillation counter.

III.2.9 Protoplast isolation

Protoplasts were isolated according to the procedure given by Nishimura et al. (1987) for sycamore suspension cultured cells, with the following modifications. One gram fresh weight of cells from 12-day-old cultures of control and -1.6 MPa mannitol stress treatment (Refer III.2.4, Imposition of osmotic stress treatments) were suspended for 45 min in 3 ml of fresh corresponding media containing 0.5 M sorbitol. The cells were then filtered, washed in fresh medium and suspended in the above media containing 1.5% cellulase (Trichoderma) and 0.2% pectolyase (Aspergillus), pH 5.8, in a 25 ml Erlenmeyer flask. The flasks were placed in an orbital shaker at 100 rpm, for 3 h. The cell aggregates were separated from the protoplasts by centrifugation in a 15 ml centrifuge tube at 10x *g* for 3 min, after the addition of 10 ml of respective protoplast isolation media without the enzymes.

III.2.10 Statistical analysis

The data were statistically analyzed by the analysis of variance (ANOVA) procedure and least significant difference (LSD, $P<0.05$) was used for mean comparisons.

III.2.11 Materials

Glycosides of cyanidin and pelargonidin were extracted from rose petals, and delphinidin was extracted from the outer pericarp of egg plant fruit. The extracted glycosides were hydrolyzed as outlined by Harborne (1984), to obtain the aglycones. Cyanidin 3-glucoside and cyanidin 3,5-diglucoside standards were obtained from rose petals. Soluble carbohydrate standards and reagents for carbohydrate analysis, and cellulase (*Trichoderma*) and pectolyase (*Aspergillus*) for protoplast isolation were obtained from Sigma Chemical Co., D- Mannitol (U-^{14}C) from ICN, Irvine, CA and the scintillation cocktail, 'Scintiverse E' from Fischer Scientific Co.

III.3 Results

III.3.1 Identification of anthocyanin pigment

The aglycone was identified as cyanidin and the sugar was identified as glucose by a comparison of the Rf values of cell culture anthocyanidin and sugar component of anthocyanin pigment with the aglycone and sugar standards respectively, by paper chromatography Table III-1). Distinction between glucose and galactose was achieved by overrunning the chromatogram in BBPW solvent system. Spectral comparison of cell-culture-anthocyanidin also indicated that the aglycone was cyanidin. Peroxide hydrolysis indicated that the attachment of the sugar molecule to the aglycone was in C-3 position. Finally, the anthocyanin was identified as cyanidin 3-glucoside by paper chromatography (Table III-1).

III.3.2 Culture growth stage and anthocyanin accumulation

The growth pattern of the culture followed a sigmoidal curve as indicated by the percent packed cell volume (PCV) (Figure III-1A). Anthocyanin per unit PCV continued to increase until the end of lag phase and early exponential phase, after which it decreased. The anthocyanin per unit culture volume (Figure III-1B) increased up to the mid-exponential phase, after which it decreased.

III.3.3 Effect of osmotic stress with glucose and mannitol

III.3.3.1 Growth and anthocyanin accumulation

Osmotic stress treatments caused an increase in the duration of the lag phase and a significant decrease in cell growth as indicated by a decrease in percent packed cell volume (Figure III-2). The lag phase was increased by 3 and 6 days by the lower (-1.1 MPa) and higher (-1.6 MPa) osmotic stress levels of both glucose and mannitol compared with control. A significant difference in growth between control and stress treatments occurred by day 6 and the difference increased further until the last harvest day. Stress treatments also caused a decrease in the rate of growth after the lag phase. Higher levels of each stress treatment caused greater growth suppression. However, both levels of glucose-induced osmotic stress caused greater growth suppression than the same levels of osmotic stress induced by mannitol. By day 12, osmotic stress induced by mannitol at -1.1 MPa caused 26% growth inhibition, while the same level of glucose-induced osmotic stress caused 44% growth inhibition. The growth inhibition figures for the -1.6 MPa osmotic stress treatments of mannitol and glucose for day 12 were 70% and 74%, respectively, compared with control. The rate of growth after the lag phase was lower in both mannitol stress treatments compared with the same level of glucose stress treatment. For the first 3 day period after the lag phase, culture growth rate in -1.1 MPa mannitol stress treatment was 1.33 cm^3 PCV day^{-1}, while it was 0.56 cm^3 PCV day^{-1} for the same level of glucose stress treatment.

Data on cell number per unit culture volume are presented in Figure III-3. In general, cell number followed the same trend as the packed cell volume. Cell number decreased with increasing osmotic stress levels, and cell number was greater in mannitol stress treatments compared with the same level of glucose stress treatments.

The anthocyanin content per unit packed cell volume (Figure III-4) was significantly higher in all stress treatments compared with the control and the differences between control and stress treatments became more pronounced over time. Stress treatments prolonged the duration of the increasing phase of anthocyanin levels. While the increase in anthocyanin content per unit PCV occurred until day 6 in the control, this increase occurred until day 9 in the -1.1 MPa stress treatments of glucose and mannitol, and until the last harvest day in -1.6 MPa stress treatments of both glucose and mannitol. The effect of osmotic stress on anthocyanin content was proportional to the level of stress imposed, with higher stress levels causing a greater increase in anthocyanin content per unit PCV. The differences in anthocyanin content among the stress treatments were more pronounced by day 12. After 12 days, cells stressed by glucose had significantly more anthocyanin compared with those stressed to the same level by mannitol. On day 12, the -1.1 MPa mannitol stress induced the accumulation of 320% more anthocyanin per unit PCV than control, while the same level of glucose stress induced 485% more anthocyanin accumulation than that of control. On the same harvest day, mannitol- and glucose stress treatments at -1.6 MPa induced the accumulation of 574% and 773% more anthocyanin than control cells, respectively (Figure III-4).

Percentage of pigmented cells (Figure III-5), anthocyanin content per pigmented packed cell volume (Figure III-6A) and anthocyanin content per pigmented cell (Figure III-6B) were determined for control and -1.6 MPa osmotic stress treatments of glucose and mannitol, to determine the cause for the difference in osmotic stress-induced anthocyanin content at the cellular level. On day 12, the percentage of pigmented cells, anthocyanin content per pigmented PCV and anthocyanin content per pigmented cell were significantly higher in osmotic stress treatments than in control cells. Protoplasts isolated from stressed cells were found to be smaller than those from the nonstressed cells (Figure III-7). On day 12, mannitol stress at -1.6 MPa produced significantly higher percentage of pigmented cells than the same level of glucose stress, while anthocyanin per pigmented packed cell volume and anthocyanin per pigmented cell were significantly higher in -1.6 MPa glucose stress treatment compared with the same level of mannitol stress.

III.3.3.2 Water relations

Changes in the osmotic potential of the media are shown in Figure III-8. Generally, the osmotic potential of the media increased (became less negative) with harvest time. From day 0 to day 12, maximum increase in medium osmotic potential occurred in control (0.273 MPa) compared with the stress treatments. A significant difference in medium osmotic potential between -1.1 MPa mannitol- and glucose stress treatments occurred by day 12. The medium osmotic potential in -1.1 MPa mannitol stress treatment was higher by 0.113 MPa than the same level of glucose stress treatment, on day 12. There was no significant difference in the medium osmotic potential between the -1.6 MPa glucose- and mannitol stress treatments. However, osmotic potential of the medium was slightly higher in mannitol stress treatment compared with glucose stress treatment.

Figures. III-9 and III-10 show changes in the water potentials and osmotic potentials of the cells, respectively. Both cell water potentials and osmotic potentials decreased (became more negative) due to osmotic stress treatments. Significantly lower cell water and osmotic potentials were observed in all stress treatments compared with control by day 3 and the difference was significant until the last harvest day. On the last harvest day, both the water and osmotic potentials of the cells subjected to the initial osmotic potential of -1.1 MPa with mannitol were significantly higher (by 0.313 MPa and 0.337 MPa, respectively) than those subjected to the same level of osmotic stress with glucose.

III.3.3.3 Soluble sugar and amino acid content of cells

The soluble sugar content of the cells increased on day 6 in both control and stress treatments (Figure III-11). While the soluble sugar content continued to increase until the last harvest day in stress treatments, it decreased on day 12 in control. Differences in soluble sugar content between control and stress treatments were observed on day 6, and the difference increased further on days 9 and 12. Among the stress treatments, cells stressed with glucose at -1.1 MPa had the highest soluble sugar content [4.47 mg $(\text{cm}^3 \text{ PCV})^{-1}$], while those stressed with mannitol at -1.1 MPa had the lowest [2.82 mg $(\text{cm}^3 \text{ PCV})^{-1}$], on day 12.

The free amino acid content of the cells decreased slightly on day 3 (Figure III-12). It increased on day 9 and continued to increase until the last harvest day, in both the control and stress treatments. Significantly higher amino acid content was observed in all the stress treatments compared with control, on day 12. The -1.1 MPa stress treatments of both mannitol- and glucose-induced significantly higher amino acid accumulation in the cells than the -1.6 MPa stress treatments, on day 12. On the same harvest day, cells subjected to osmotic stress with glucose at -1.1 MPa produced significantly higher amino acid levels [2.08 mg (cm^3 PCV)$^{-1}$] than those stressed with mannitol [1.65 mg (cm^3 PCV)$^{-1}$] at the same stress level.

The ratio of soluble sugar to amino acid is presented in Figure III-13. This ratio increased on day 3 in all the treatments. The ratio was higher in all the stress treatments compared with control throughout the harvest time. Cells subjected to osmotic stress with glucose at -1.1 MPa showed significantly higher ratio of soluble sugar to amino acids than those subjected to the same level of stress with mannitol on day 12.

III.3.3.4 Soluble carbohydrate composition

Because the cells were subjected to osmotic stress with glucose and mannitol, variation in the cellular content of soluble carbohydrates was expected. Because the difference in the osmotic potential and anthocyanin content of the cells among the stress treatments became pronounced on the last harvest day, the soluble carbohydrate composition of cells was determined for day 12 (Table III-2).

The glucose content of the cells was higher in all the stress treatments than in the control. Cells subjected to both levels of glucose stress accumulated significantly higher levels of glucose than those subjected to the same level of mannitol stress. No significant difference in the cellular glucose content was observed between the two tested levels of mannitol stress or between the two levels of glucose stress.

Although a general increase in the cellular fructose content was observed in all the stress treatments compared with control, significantly higher [74.0 nmol (cm^3 PCV)$^{-1}$] fructose level was observed in -1.1 MPa glucose stress treatment compared with both control cells and the remaining stress treatments.

Sucrose content in the cells of all stress treatments was significantly higher than that of the control. The sucrose content of cells increased with the increase in the level of mannitol stress, but no such relationship was observed between the glucose stress levels and sucrose content. Glucose at -1.1 MPa [684 nmol $(cm^3\ PCV)^{-1}$] and mannitol at -1.6 MPa [678 mmol $(cm^3\ PCV)^{-1}$] induced significantly higher amount of sucrose accumulation in the cells than the remaining treatments.

The inositol content of the cells was significantly higher in all the stress treatments compared with that of the control. Cells stressed with -1.1 MPa glucose had the highest amount of inositol [119.4 nmol $(cm^3\ PCV)^{-1}$], which was significantly higher than the remaining treatments. No significant difference in the inositol content of cells was observed between the two mannitol stress levels.

To determine whether the cells accumulate mannitol, the mannitol content of the cells was determined on day 12. The mannitol stressed cells were found to accumulate mannitol to a greater extent. At the high level of mannitol stress, a greater amount of mannitol was accumulated (Table III-2). As mannitol was found to accumulate in the cells, its metabolism was determined by feeding D-mannitol $(U\text{-}^{14}C)$ on day 12. Radioactivity was detected in the carbon dioxide evolved from the cells. Cells of both -1.1 MPa [0.022 μmol mannitol h^{-1} (mg cell dry weight)$^{-1}$] and -1.6 MPa [0.116 μmol mannitol h^{-1} (mg cell dry weight)$^{-1}$] mannitol stress treatments were found to metabolize mannitol. However, most of the mannitol was found to be present in the media on the last harvest day [212 μmol ml^{-1} out of an initial level of 257 μmol ml^{-1} in -1.1 MPa treatment (82%) and 459 μmol ml^{-1} from an initial level of 498 μmol ml^{-1} in -1.6 MPa treatment (92%). Therefore, it is possible that the cells needed a period of adaptation before utilizing mannitol as an energy source and hence metabolism of mannitol by these cells might have occurred only in the later days of culture period.

III.3.4 Effect of osmotic stress with polyethylene glycol

When the cells were subjected to osmotic stress with PEG (MW 3350) at 0, 30, 60 and 75 mM concentration, corresponding to osmotic potentials of -0.6, -0.75, -1.25 and -1.5 MPa, a significant increase in the anthocyanin content of the cells occurred with decreasing medium osmotic potentials (Figure III-14).

III.4 Discussion

The anthocyanin pigment produced in this cell culture was identified to be cyanidin 3-glucoside. All the anthocyanins identified in different species and hybrids of poplar were found to be derivatives of cyanidin including cyanidin 3-glucoside (Bendz & Haglund 1968; Chang et al. 1989). Matsumoto et al. (1973) identified the anthocyanin pigment produced in the tissue culture of a hybrid poplar as cyanidin 3-glucoside.

Most plant cell cultures produce maximum levels of secondary metabolites in the stationary phase of culture growth (Mantel & Smith 1983), while only a few are known to produce maximum levels of secondary metabolites in the lag phase (Noguchi & Sankawa 1982) as observed in the present cell culture. Unlike the observations made by Do & Cormier (1990) who observed two peaks of anthocyanin accumulation in grape cell suspension culture, one in the lag phase and the other in the stationary phase, the cell culture in the present study accumulated anthocyanin only in the lag phase (Figure III-1A). Accumulation of anthocyanin in the lag phase and the decrease in anthocyanin level subsequent to higher growth rate indicates an inverse relationship between growth and secondary metabolism in this culture. Such relationship has been observed in most cell cultures (Sakuta & Komamine 1987).

The decrease in anthocyanin content per unit culture volume after 12 days (Figure III-1B) suggests that anthocyanin degradation occurred in this culture during the rapid culture growth stage. Therefore, it is possible that the cell utilized the degraded anthocyanin for its energy needs. It has been reported that phenolic monomers can be readily degraded by plants, so carbon stored in them could be mobilized when needed (Gershenzon 1984). It should, however, be noted that because phenolic compounds are more oxidized than carbohydrates, they are not rich in stored energy. Also, because anthocyanin is a high molecular weight compound than sugars and amino acids, it is not an ideal osmotic solute (Borowitzka 1981). Because anthocyanin is accumulated in the vacuole and is positively charged at acidic pH, it might be accumulated in place of cations. Hence, anthocyanin accumulation in this cell culture appears to be an unavoidable consequence of growth inhibition and associated physiological changes, or it is accumulated to serve several different functions such as reserve energy source, osmotic solute and charge balance in the vacuole though it is not efficient in any of them.

Osmotic stress-induced inhibition of cell growth in this culture appears to be due to the inhibition of cell division (Figure III-3). Inhibition of cell division by osmotic stress in cell suspension culture has been previously reported (Do & Cormier 1991). In the present study, osmotic stress resulted in an increase in the anthocyanin content of the cells (Figure III-4). An inverse relationship between growth and secondary metabolite accumulation has been demonstrated using inhibitors of cell division (Lindsey & Yeoman 1985) and under osmotic stress conditions (Do & Cormier 1990). This inverse relationship might be due to the diversion of metabolites from primary metabolism to secondary metabolism under growth suppressing conditions (Fowler 1986). In the present study, both levels of initial osmotic stress induced by glucose caused greater growth inhibition and higher anthocyanin content in the cells compared with the same level of mannitol stress. The difference in anthocyanin content of the cells between glucose- and mannitol stress treatments became apparent only after the initiation of cell growth. Hence, the difference in the effect of glucose- and mannitol stress on anthocyanin content of cells is perhaps due to the difference in their effect on cell growth.

Cellular heterogeneity is a common feature of plant cell cultures (Hall & Yeoman

1986). An increase in the anthocyanin content of a unit volume or weight of cells could occur due to an increase in the proportion of cells accumulating anthocyanin or due to an increase in the anthocyanin concentration in anthocyanin producing cells. Determination of the percentage of pigmented cells (Figure III-5) and the anthocyanin content per pigmented PCV (Figure III-6A) indicated that both are responsible for the higher anthocyanin content of a unit volume of cells brought about by osmotic stress. Anthocyanin per pigmented PCV is taken as an indicator of anthocyanin concentration in pigmented cells. The increase in the anthocyanin content per unit pigmented PCV by osmotic stress is not merely due to the decrease in the cell size brought about by osmotic stress conditions (Figure III-7), but is due to the actual increase in the anthocyanin content of the anthocyanin producing cells as indicated by the increase in the anthocyanin per pigmented cell (Figure III-6B). There appears to be a great scope for increasing the anthocyanin production in this cell culture by increasing the proportion of productive cells and also by increasing the concentration of anthocyanin in pigmented cells, because a large proportion of cells still did not accumulate anthocyanin even in the treatments which produced high anthocyanin levels and large variation occurred in anthocyanin content among the pigmented cells (visual observation). The presence of cell aggregates might be one of the reasons for the cellular heterogeneity with respect to anthocyanin accumulation observed in this culture because the cells might be subjected to different degree of stress.

A comparison of the effects of glucose- and mannitol stress indicated that an increase in the proportion of cells accumulating anthocyanin is largely responsible for the higher anthocyanin level in mannitol stressed cells (Figure III-5), while increase in the concentration of anthocyanin in the pigmented cells is responsible for the higher anthocyanin content of glucose stressed cells (Figure III-6A & 6B). The difference in anthocyanin concentration between mannitol- and glucose stressed cells became apparent only after the resumption of cell growth. The greater growth inhibition caused by glucose stress might have resulted in increased anthocyanin concentration in the cells. The cause for the presence of a higher proportion of anthocyanin accumulating cells in mannitol stressed cells than the glucose stressed cells is not known.

The greater increase in medium osmotic potential from day 0 to day 12 observed in the control (Figure III-8) was perhaps due to the higher cell growth observed in the controls compared with the stress treatments. Because of higher cell growth, more nutrients were probably taken up by the cells from the medium, resulting in a greater increase in the medium osmotic potential. Perhaps, for the same reason, the medium osmotic potential in -1.1 MPa mannitol stress treatment was higher than the same level of glucose stress treatment.

Where sucrose was used as a source of sugar, an initial decrease in the osmotic potential of medium has been reported (Do & Cormier 1990) while no such decrease in medium osmotic potential occurred in our experiment because glucose was used as the carbon source. Therefore, in studies on the effect of a particular osmotic potential of media, it is preferable to use glucose to sucrose as the carbon source in cell cultures where possible, to avoid variations in osmotic potential occurring due to sucrose hydrolysis.

The decreased water potential of the cells (Figure III-9) appears to be responsible for the reduced growth and higher anthocyanin content of the cells subjected to osmotic stress. Water stress is known to decrease growth and increase secondary metabolite levels (Gershenzon 1984). Differences in the water potentials and osmotic potentials of cells (Figures III-9 & III-10 respectively) between the same initial osmotic stress levels induced by glucose and mannitol occurred only after the commencement of cell growth (Figure III-2). An increase in the osmotic potential of the medium occurring due to higher growth and accompanied higher uptake and utilization of nutrients for cell growth, might be the reason for the higher water potentials and osmotic potentials of the cells stressed with mannitol. This becomes evident by a comparison of the medium osmotic potential (Figure III-8), cell water potential (Figure III-9) and cell osmotic potential (Figure III-10) between -1.1 MPa glucose- and mannitol stress treatments. Therefore, the difference in the cell water potentials and osmotic potentials between glucose- and mannitol stress treatments occurred due to the difference in the effect of these osmotica on cell growth.

The osmotic potentials of the cells also decreased in those treatments which decreased the cell water potentials, indicating that the cells were subjected to water stress. Accumulation of osmotic solutes might influence anthocyanin levels directly, or indirectly through their influence on cell growth. Reduction of cell growth in turn, might result in the accumulation of certain solutes (eg. accumulation of certain amino acids due to inhibition of protein synthesis) that might increase anthocyanin production (Gershenzon 1984).

Sugars and amino acids are the important groups of solutes accumulated by plant cells during osmoregulation (Borowitzka 1981). An increase in the soluble sugar and amino acid content of the cells occurred due to osmotic stress treatments. This might be responsible for the reduction in the osmotic potential, decreased cell growth and increased anthocyanin production of stressed cells compared with control. However, among the stress treatments the amount of these solutes could not be related to the osmotic potentials of cells or anthocyanin levels.

It has been reported that increased supply of sugars and decreased supply of nitrogen through the media caused a reduction in cell growth and an increase in anthocyanin levels in grape cell suspension culture (Do & Cormier 1991). Therefore, it appears that the amount of soluble sugars and free amino acids relative to one another might also be important in influencing growth and anthocyanin accumulation, in addition to their individual quantities. Not only was an increase in the ratio of soluble sugars to free amino acids observed in all the stress treatments compared with control, this ratio was related to the anthocyanin levels among the stress treatments. Increase in this ratio might result in an imbalance in the solute composition of the cells which might inhibit cell growth and increase anthocyanin production. It is possible that this effect of osmotic stress is similar to the dilution effect (Simpkin & Street 1970), in that, it increases the ratio of soluble sugars to free amino acids due to the leakage of amino acids to the medium and due to the uptake of sugars from the medium upon inoculation in a fresh medium. However, it is unclear how this increase in the ratio of soluble sugars to free amino acids might result in growth suppression and higher anthocyanin production.

A significantly higher ratio of soluble sugars to amino acids in the -1.1 MPa glucose stress treatment compared with the same level of mannitol stress treatment on day 12 could be the reason for the difference in growth and anthocyanin levels observed between these two treatments. The nonsignificant difference in the soluble sugar to amino acid ratio between -1.6 MPa mannitol stress treatment and the same level of glucose stress treatment did not, however, relate to the differences in cell growth and anthocyanin levels. Subsequent analysis of soluble carbohydrate composition of the cells showed that sucrose was the major soluble sugar present in stressed cells (Table III-2) and that the sucrose content of cells stressed with -1.6 MPa mannitol was higher than that of -1.6 MPa glucose stress treatment on day 12. Thus, the reason for the higher ratio of soluble sugar to amino acid found in cells stressed at -1.6 MPa by mannitol compared with those stressed to the same level by glucose could be because of the higher values for sucrose than for monosaccharides obtained by the anthrone method (Ashwell 1957), which was used for soluble sugar estimation.

The cellular content of glucose, fructose, sucrose and inositol was higher in all the stress treatments compared with control on day 12 (Table III-2), when differences in the osmotic potential and anthocyanin content of the cells became most pronounced. Therefore, a general increase in all of these individual sugars contributed to the increase in the soluble sugars, decrease in the osmotic potentials and hence decreased growth and increased anthocyanin content of the stressed cells. However, among the stress treatments, no direct correlation was found between the sugar composition and anthocyanin content of the cells.

Mannitol was taken up by the cells and metabolized. However, the presence of most of the mannitol in the medium on day 12 indicated that mannitol was not taken up by these cells in substantial amounts. Therefore, comparison of the osmotic effects of glucose and mannitol in this culture is in fact a comparison between an osmoticum that is readily taken up and metabolized (glucose) and an osmoticum that is slowly taken up (mannitol) (Thompson et al. 1986) and metabolized. In a cell culture accumulating maximum anthocyanin in the lag or early exponential phase, carbon source will not be limiting in the medium at the time of maximum anthocyanin accumulation. Therefore, the different degree of metabolizable nature of glucose and mannitol may not have influenced anthocyanin production in this culture. An attempt to determine the effect of deoxyglucose, a nonmetabolizable form of glucose at osmotic potentials of -1.1 MPa and -1.6 MPa in control medium was not possible due to the toxicity of deoxyglucose to the cells (data not shown).

Polyethylene glycol with a molecular weight greater than 3000 is not absorbed by plant cells (Mextal et al. 1975). When cells were stressed with various concentrations of PEG of MW 3350, which served as an external osmoticum, a significant increase in anthocyanin production with decreasing medium osmotic potential was observed (Figure III-14). Thus, PEG induced anthocyanin accumulation indicated that anthocyanin accumulation in this culture was enhanced by osmotic stress and in fact was not due to the presence of higher amount of carbon source in the medium.

Mannitol is found to be less inhibitory to enzymes and hence is considered as a compatible solute (Borowitzka 1981). Therefore, accumulation of mannitol in the mannitol stressed cells might have resulted in higher cell growth and consequently, lower anthocyanin levels compared with those stressed to the same level by glucose.

Significant differences in cell water potential, osmotic potential, soluble sugar to amino acid ratio and anthocyanin content between glucose- and mannitol stress treatments occurred subsequent to growth initiation. Therefore, solute composition just before the commencement of growth might play an important role in influencing the growth rate of glucose- and mannitol stressed cells. The mannitol content of the stressed cells was determined for the -1.1 MPa mannitol- and glucose stress treatments, on day 6, the time just before the commencement of growth in these two treatments. The cells stressed to -1.1 MPa with mannitol were found to accumulate mannitol [75 nmol (cm^3 PCV)$^{-1}$] on day 6. This observation, again indicated that the presence of mannitol, a compatible solute, in mannitol stressed cells might be responsible for the difference in growth and hence anthocyanin levels observed between the glucose- and mannitol stress treatments.

Anthocyanin accumulation in this culture is not a stress shock response because the culture continued to accumulate high anthocyanin levels on repeated subculturing in the low osmotic potential medium.

Both the extent of osmotic stress and the type of solute used to induce osmotic stress appear to influence anthocyanin levels in this culture through their influence on cell growth. Osmotically induced growth inhibition could cause an inhibition of primary metabolism and consequent diversion of metabolites for anthocyanin biosynthesis. The solutes accumulated due to growth inhibition might, in turn, influence anthocyanin production by inducting the activity of the regulatory enzymes of anthocyanin biosynthesis. Hence, an understanding of the cellular composition of other groups of solutes which are known to increase due to growth inhibition under osmotic stress condition, such as amino acids and organic acids and the activity of the regulatory enzymes of anthocyanin biosynthesis might be helpful in the further elucidation of the mechanism of osmotically induced anthocyanin production. These aspects are dealt with in the next chapter.

Table III-1. Paper chromatographic Rf values (x 100) of cell culture anthocyanidin, sugar component of anthocyanin, anthocyanin pigment and standards in different solvent systems.

Anthocyanidin source	Solvent system		
	Forestal	Formic acid	BAW
Cell culture	52	18	64
Cyanidin	52	18	66
Pelargonidin	68	30	77
Delphinidin	33	13	41

Sugar source	Solvent system		
	BAW	BEW	BBPW (Rg, Overrun)
Cell culture	42	40	1.00
Glucose	42	39	1.00
Galactose	40	38	0.88
Arabinose	47	47	------
Rhamnose	62	68	------
Xylose	51	56	-------

Anthocyanin source	Solvent system		
	BAW	BuHCl	1% HCl
Cell culture	19	39	7
Cyanidin 3-glucoside	18	40	7
Cyanidin 3,5-diglucoside	12	9	16

Note: The chromatogram in BBPW solvent system was overrun to separate glucose and galactose. Rg values were calculated for the overrun chromatogram in BBPW solvent system with reference to glucose, taking the Rg value for glucose as 1.0.

Solvent system composition: Forestal= Conc. HCl:acetic acid:water (3:30:10); Formic acid= Conc. HCl:formic acid:water (2:5:3); BAW= butanol:acetic acid:water (4:1:5); BEW= butanol:ethanol:water (4:1:2.2); BBPW= butanol:benzene:pyridine:water (5:1:3:3); BuHCl= butanol:2M HCl (1:1).

Table III-2. Amount [nmol (cm^3 PCV)$^{-1}$] of various soluble carbohydrates 12 days after culture inoculation in cultures subjected to osmotic stress treatments. Treatments were imposed as described in Figure III-2. Values are means of three replicates. Values in parenthesis are +/- standard error. F test was significant at $P<0.01$ for all the soluble carbohydrates. Least significant difference (LSD, $P<0.05$) values for mean comparison are presented.

Treatment	Soluble carbohydrate				
	Glucose	Fructose	Sucrose	Inositol	Mannitol
Control	8.72	18.55	24.64	19.50	0.00
(-0.6 MPa)	(2.37)	(6.37)	(16.00)	(3.45)	
Glucose	110.58	74.83	684.16	119.43	0.00
-1.1 MPa	(28.23)	(11.10)	(35.80)	(4.40)	
Glucose	104.86	30.25	540.85	53.67	0.00
-1.6 MPa	(5.38)	(2.32)	(65.40)	(6.88)	
Mannitol	46.96	35.40	299.53	89.61	424.67
-1.1 MPa	(10.55)	(0.60)	(27.24)	(10.86)	(56.70)
Mannitol	58.56	30.31	677.66	94.85	656.78
-1.6 MPa	(8.24)	(2.47)	(49.98)	(8.89)	(103.53)
LSD	44.80	18.68	134.11	23.40	166.36

Figure III-1. Changes in cell growth (A) and anthocyanin content (A and B) of poplar cell suspension at different days after culture inoculation. Cultures were inoculated in MS medium containing 3% sucrose, 2 μM 2,4-D and 1 μM kinetin at an inoculum density of 5% packed cell volume. Each point is the mean of three replicates. Error bars represent +/- SEM.

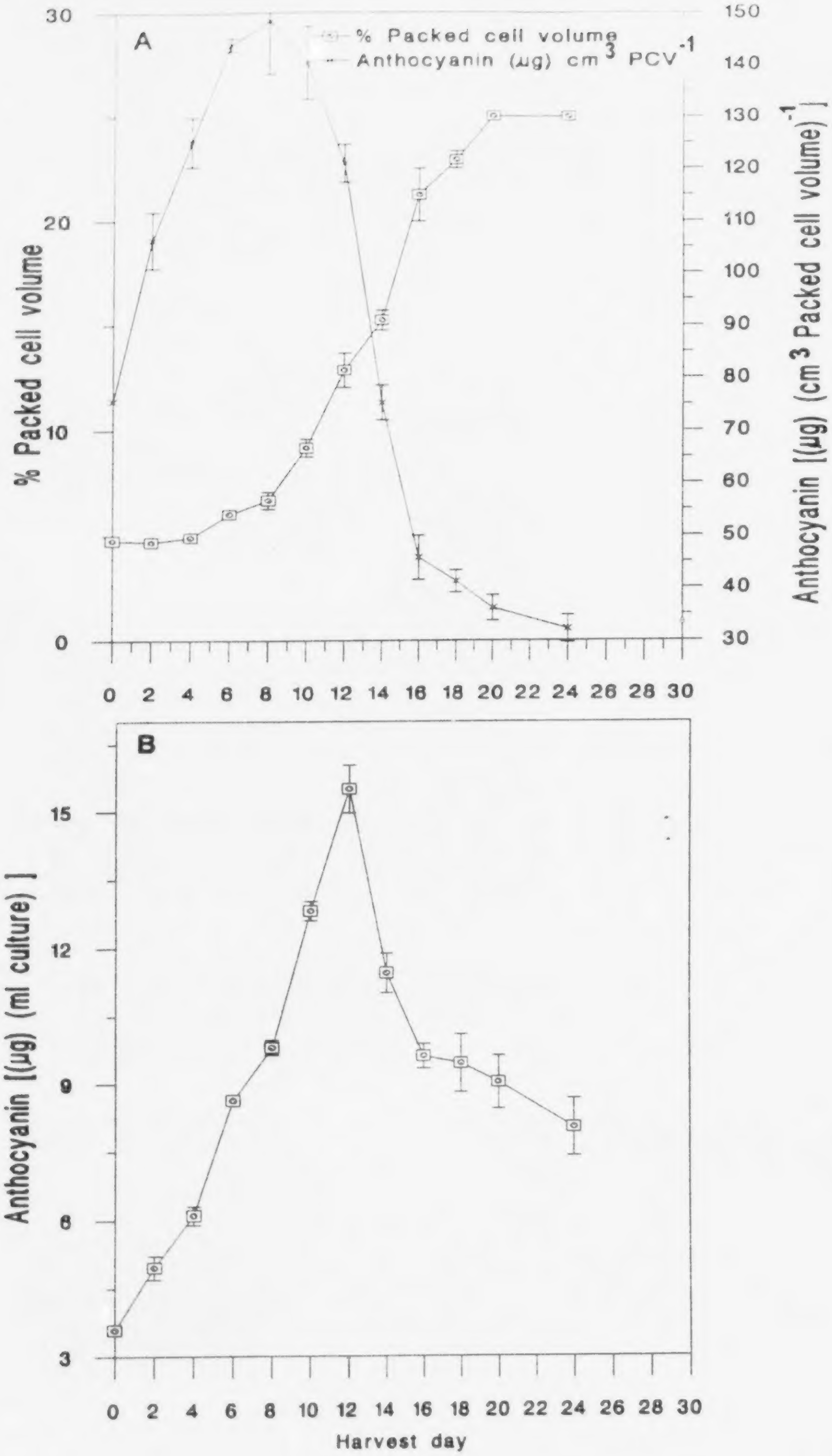

A
% Packed cell volume
Anthocyanin (µg) cm3 PCV-1
% Packed cell volume
Anthocyanin [(µg) (cm3 Packed cell volume)-1]
0
10
20
30
30
40
50
60
70
80
90
100
110
120
130
140
150
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
B
Anthocyanin [(µg) (ml culture)-1]
3
6
9
12
15
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Harvest day

Figure III-2. Growth of cell cultures subjected to osmotic stress treatments at different days during culture. Cells were subjected to osmotic stress by inoculating the culture in a medium of low osmotic potential. A medium osmotic potential of -1.1 MPa and -1.6 MPa was obtained by the addition of either glucose or mannitol to the basic MS medium which contained 105 mM glucose (medium osmotic potential of -0.6 MPa), which served as the control. Each point is the mean of three replicates. Error bars represent +/- standard error of the mean (SEM). F test for osmotic stress treatments, harvest day and their interactions were significant at $P< 0.01$. Least significant difference (LSD, $P<0.05$) for mean comparison = 1.39.

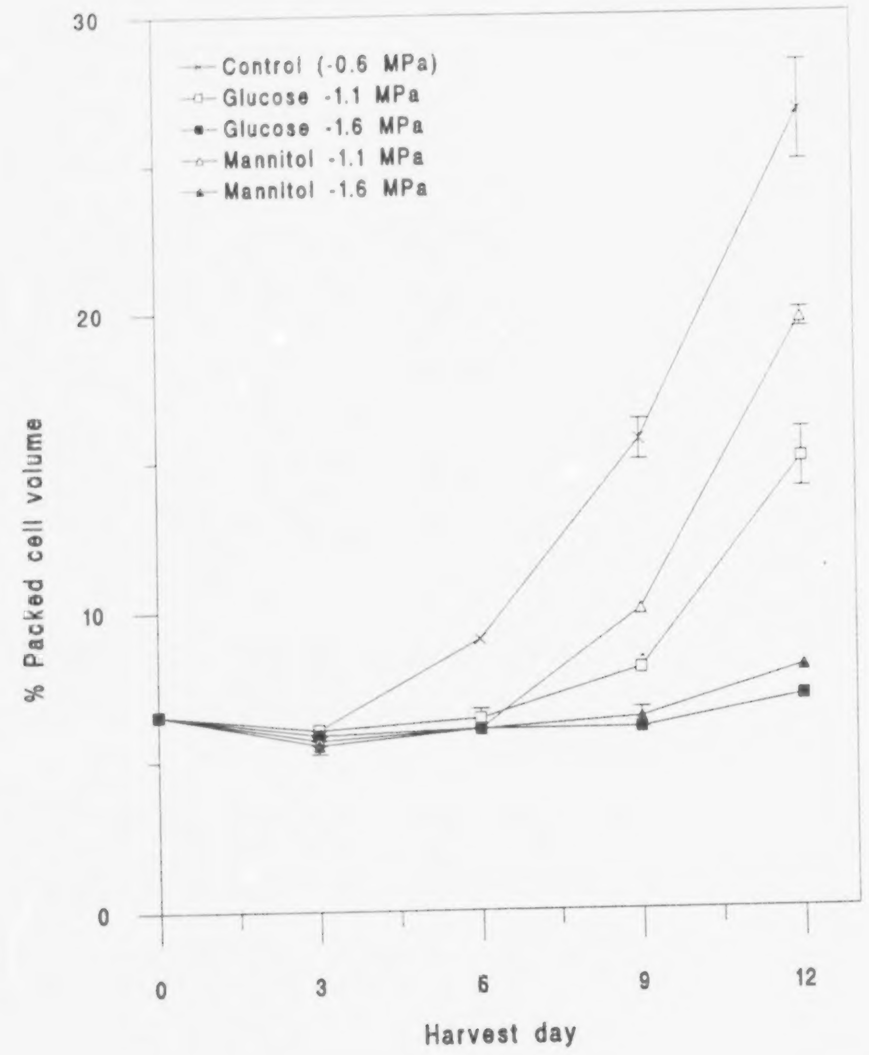

30
20
10
0
% Packed cell volume
0
3
6
9
12
Harvest day
Control (-0.6 MPa)
Glucose -1.1 MPa
Glucose -1.6 MPa
Mannitol -1.1 MPa
Mannitol -1.6 MPa

Figure III-3. Changes in cell number per unit culture volume at different days after culture inoculation, in cultures subjected to osmotic stress treatments. Treatments were imposed as described in Figure III-2. Each point is the mean of three replicates. Error bars represent +/- SEM. F test for osmotic stress treatments, harvest day and their interactions were significant at $P < 0.01$. LSD ($P<0.05$) for mean comparison = 0.95.

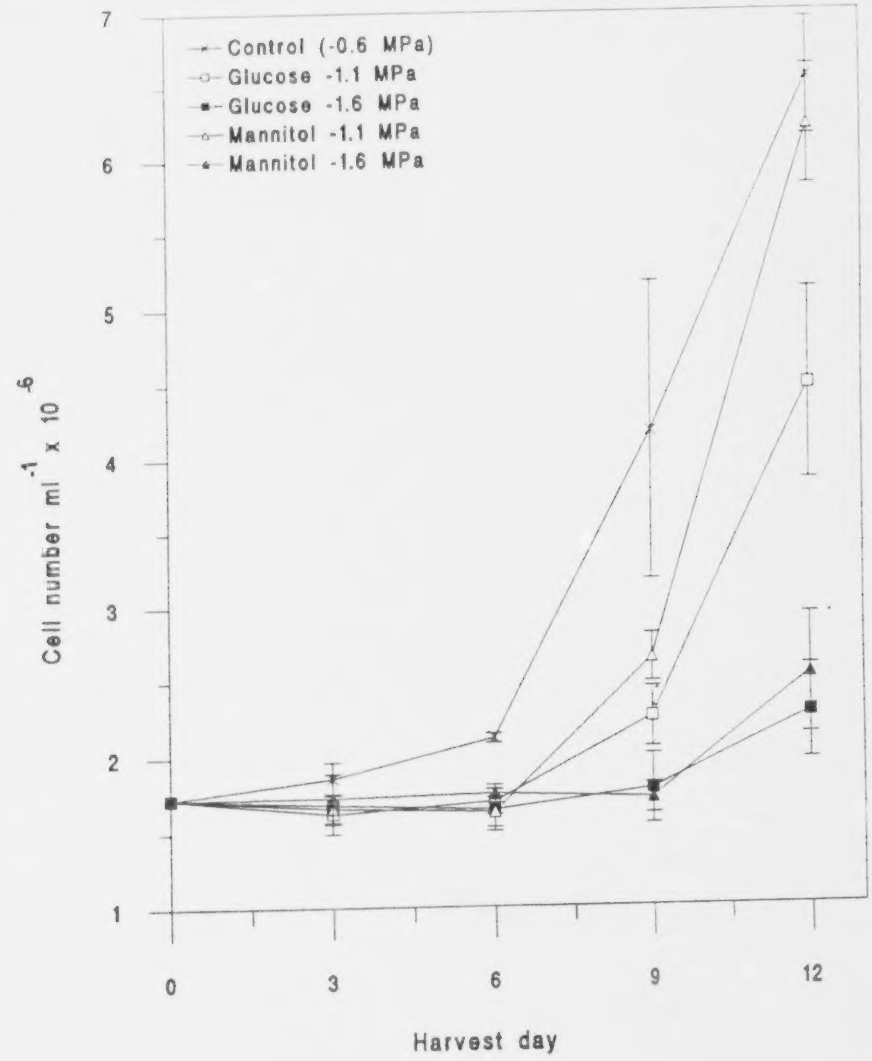

Control (-0.6 MPa)
Glucose -1.1 MPa
Glucose -1.6 MPa
Mannitol -1.1 MPa
Mannitol -1.6 MPa
Cell number ml^{-1} x 10^{-6}
1
2
3
4
5
6
7
0
3
6
9
12
Harvest day

Figure III-4. Anthocyanin content per unit packed cell volume (PCV) at different days after culture inoculation, in cultures subjected to osmotic stress treatments. Treatments were imposed as described in Figure III-2. Each point is the mean of three replicates. Error bars represent +/- SEM. F test for osmotic stress treatments, harvest day and their interactions were significant at $P< 0.01$. LSD ($P<0.05$) for mean comparison = 41.2.

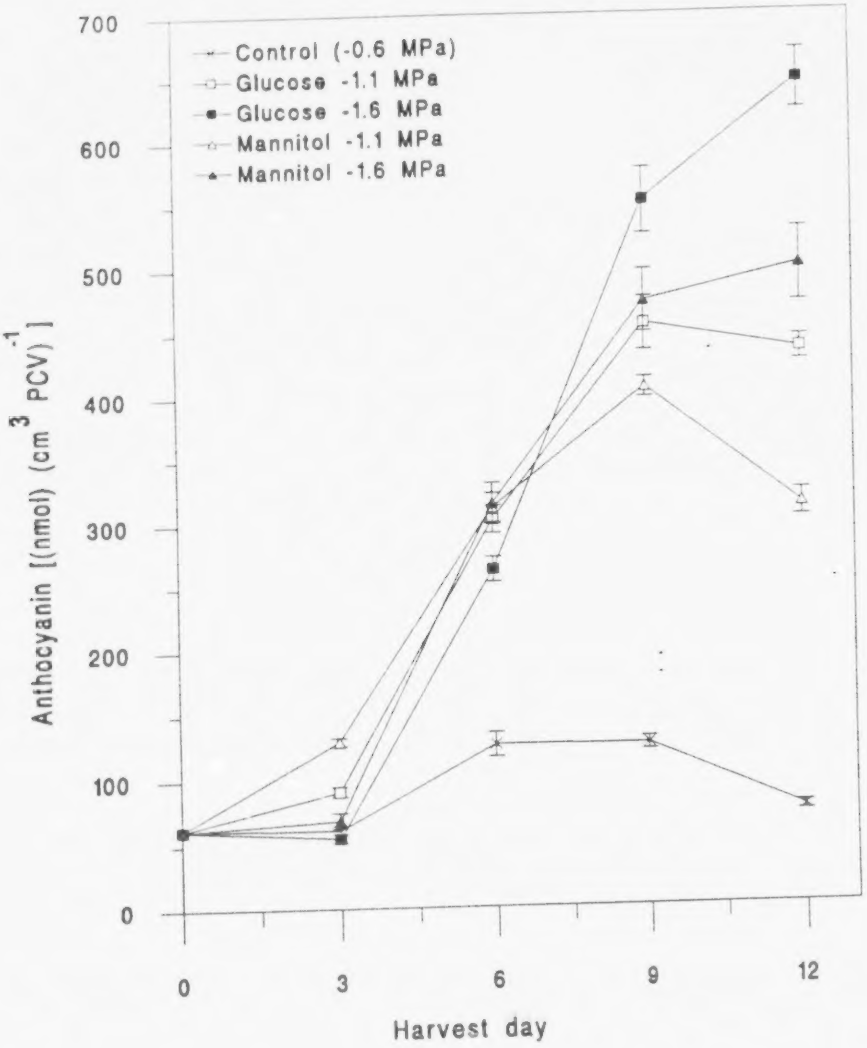

Control (-0.6 MPa)
Glucose -1.1 MPa
Glucose -1.6 MPa
Mannitol -1.1 MPa
Mannitol -1.6 MPa
Anthocyanin [(nmol) (cm^3 PCV)$^{-1}$]
0
100
200
300
400
500
600
700
Harvest day
0
3
6
9
12

Figure III-5. The percentage of pigmented cells at different days after culture inoculation in cultures subjected to osmotic stress treatments. Treatments were imposed as described in Figure III-2. Each point is the mean of three replicates. Error bars represent +/- SEM. F test for osmotic stress treatment and harvest day were significant at $P < 0.01$, and for their interaction the F test was significant at $P<0.05$. LSD ($P<0.05$) for mean comparison = 6.26.

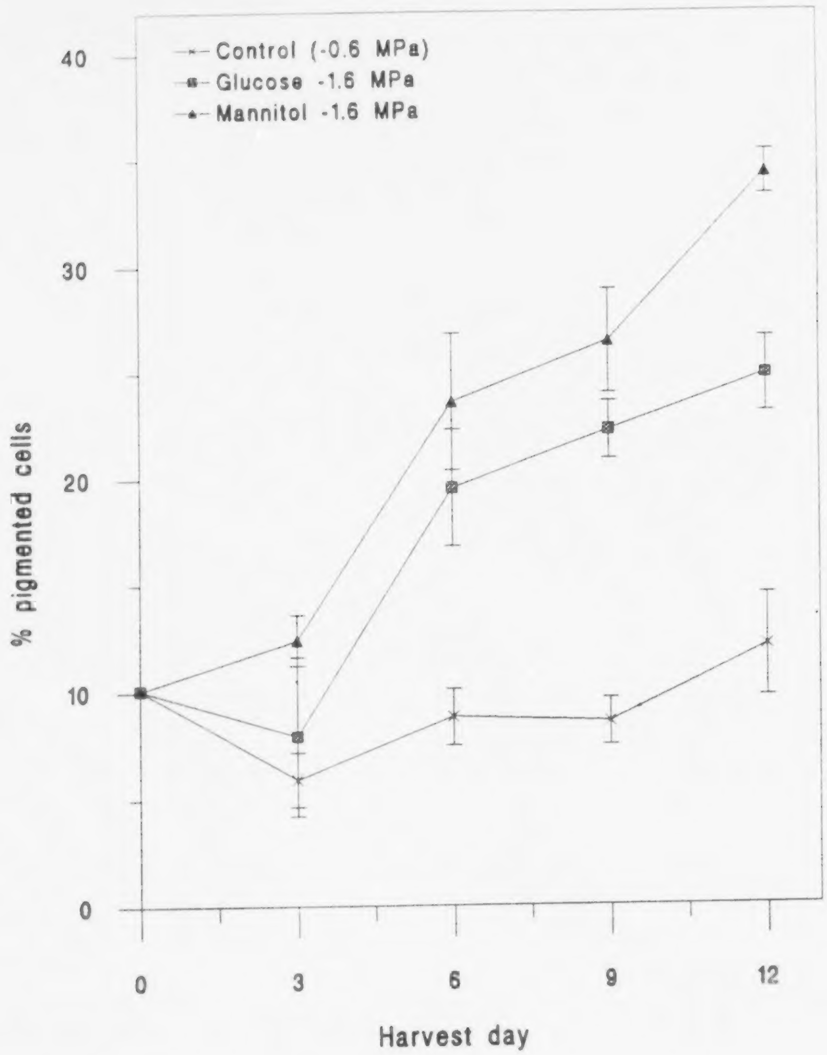

Control (-0.6 MPa)
Glucose -1.6 MPa
Mannitol -1.6 MPa
40
30
20
10
0
% pigmented cells
0
3
6
9
12
Harvest day

Figure III-6. Anthocyanin content per unit pigmented packed cell volume (PCV) (A) and anthocyanin content per pigmented cell (B) at different days after culture inoculation in cultures subjected to osmotic stress treatments. Treatments were imposed as described in Figure III-2. Each point is the mean of three replicates. Error bars represent +/- SEM.

A) F test for osmotic stress treatment, harvest day and their interaction were significant at $P < 0.01$. LSD ($P < 0.05$) for mean comparison = 0.511.

B) F test for osmotic stress treatment, harvest day and their interaction were significant at $P < 0.01$. LSD ($P < 0.05$) for mean comparison = 20.22

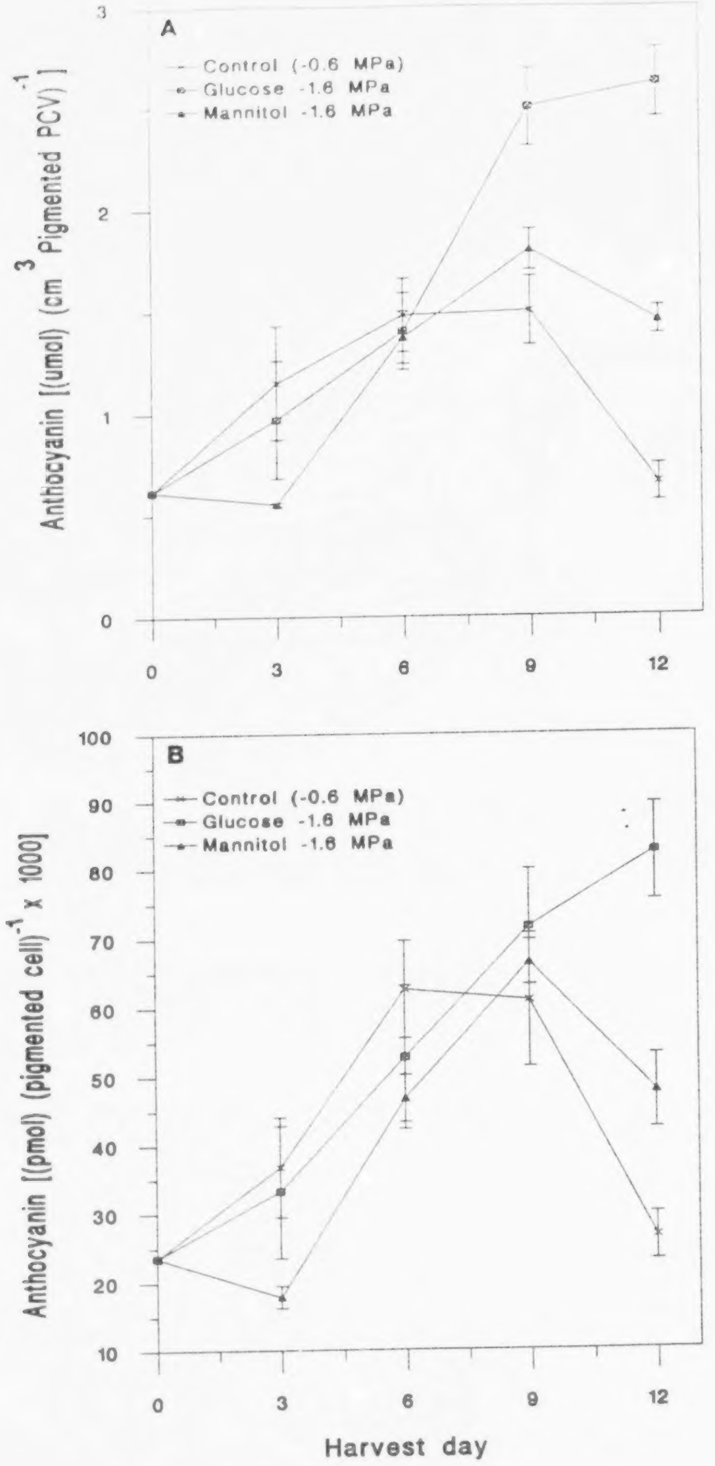

A
Control (-0.6 MPa)
Glucose -1.6 MPa
Mannitol -1.6 MPa
Anthocyanin [(umol) (cm 3 Pigmented PCV) -1]
0
1
2
3
0
3
6
9
12
B
Control (-0.6 MPa)
Glucose -1.6 MPa
Mannitol -1.6 MPa
Anthocyanin [(pmol) (pigmented cell) -1 x 1000]
10
20
30
40
50
60
70
80
90
100
0
3
6
9
12
Harvest day

Figure III-7. Photographs of protoplasts showing the cell size difference between Control (A) and osmotically stressed (mannitol -1.6 MPa) (B) cells. Protoplasts were isolated according to the procedure given by Nishimura et al. (1987), as described in 'Materials and Methods' section. Bar represents 100 μm.

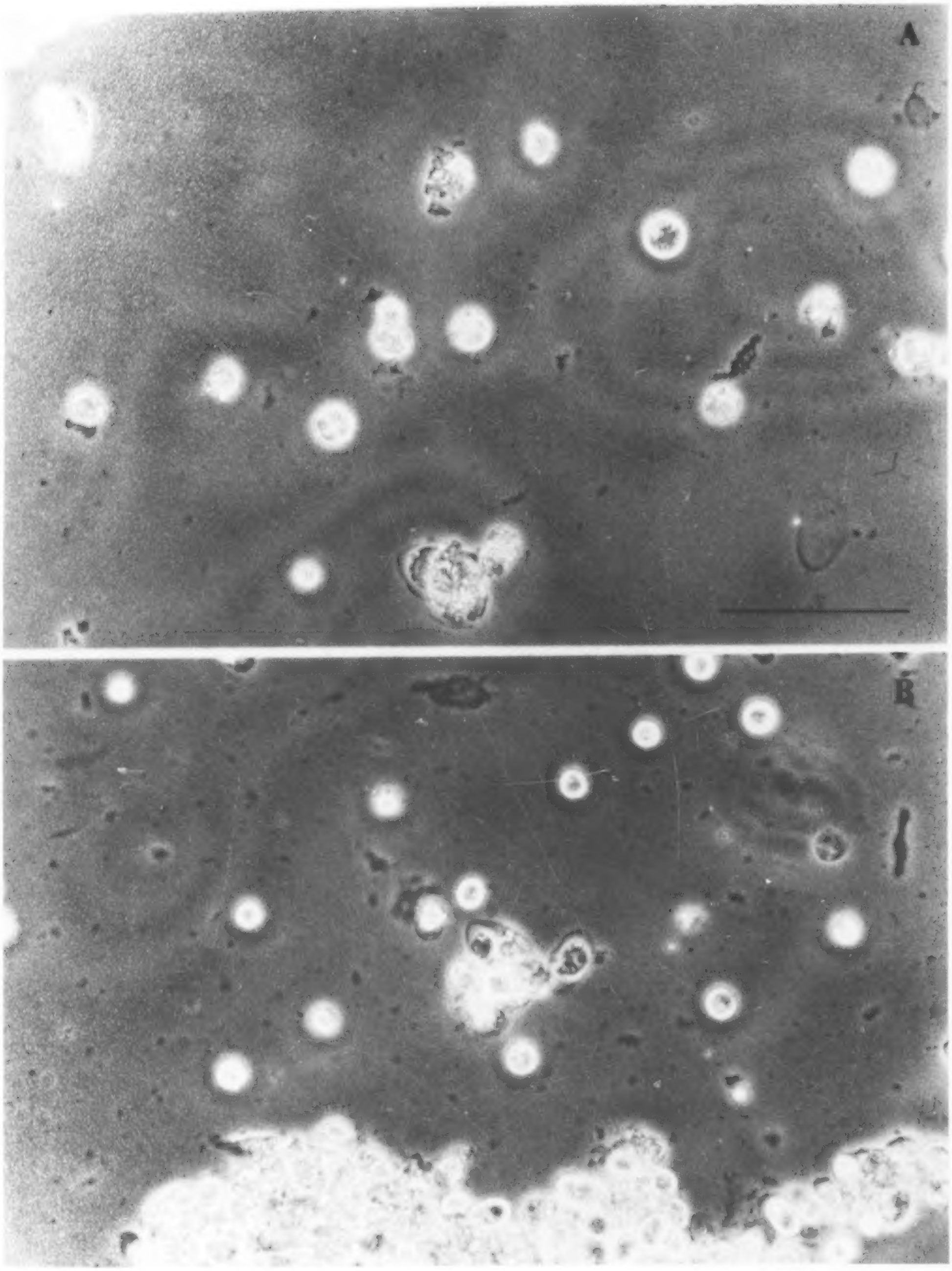

A
B

Figure III-8. Changes in the medium osmotic potential at different days after culture inoculation in cultures subjected to osmotic stress treatments. Treatments were imposed as described in Figure III-2. Each point is the mean of three replicates. Error bars represent +/- SEM. F test for osmotic stress treatment and harvest day were significant at $P < 0.01$, and for their interaction the F test was nonsignificant at $P<0.05$. LSD ($P<0.05$) for mean comparison = 0.074.

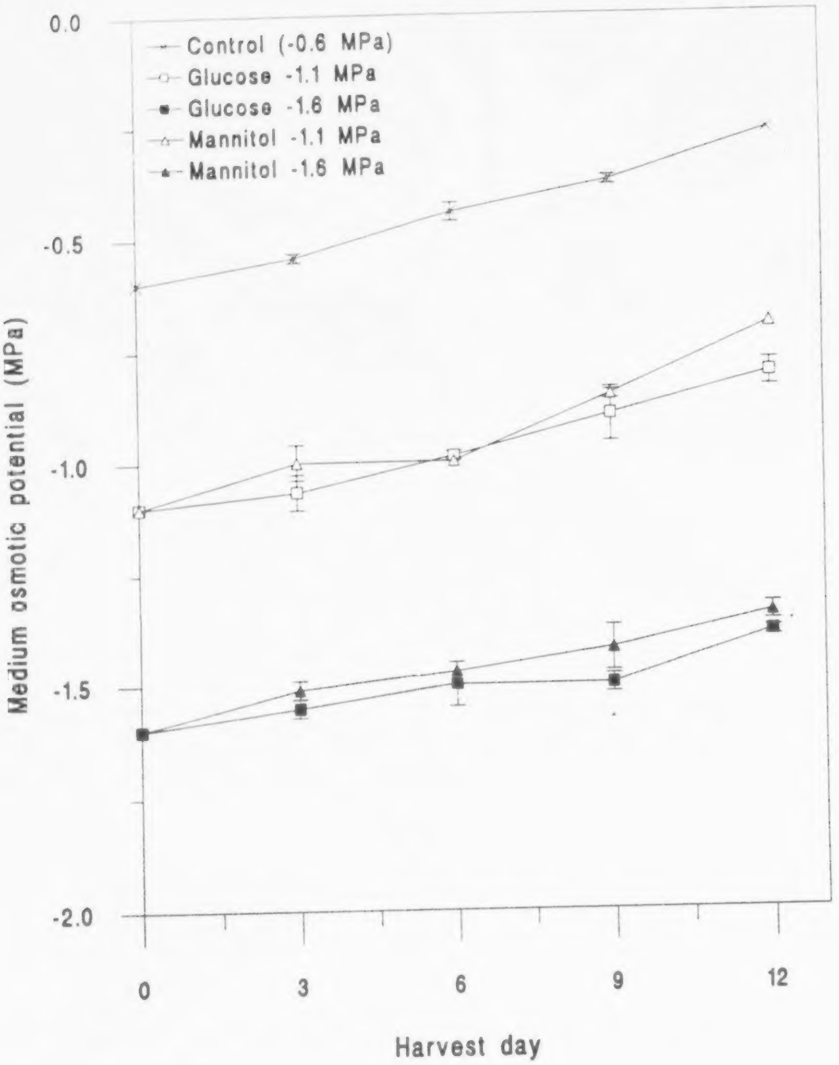

Control (-0.6 MPa)
Glucose -1.1 MPa
Glucose -1.6 MPa
Mannitol -1.1 MPa
Mannitol -1.6 MPa
0.0
-0.5
-1.0
-1.5
-2.0
Medium osmotic potential (MPa)
0
3
6
9
12
Harvest day

Figure III-9. Changes in the cell water potential at different days after culture inoculation in cultures subjected to osmotic stress treatments. Treatments were imposed as described in Figure III-2. Each point is the mean of three replicates. Error bars represent +/- SEM. F test for osmotic stress treatment was significant at $P< 0.01$, for harvest day it was significant at $P<0.05$ and for their interaction it was nonsignificant at $P<0.05$. LSD ($P<0.05$) for mean comparison = 0.227.

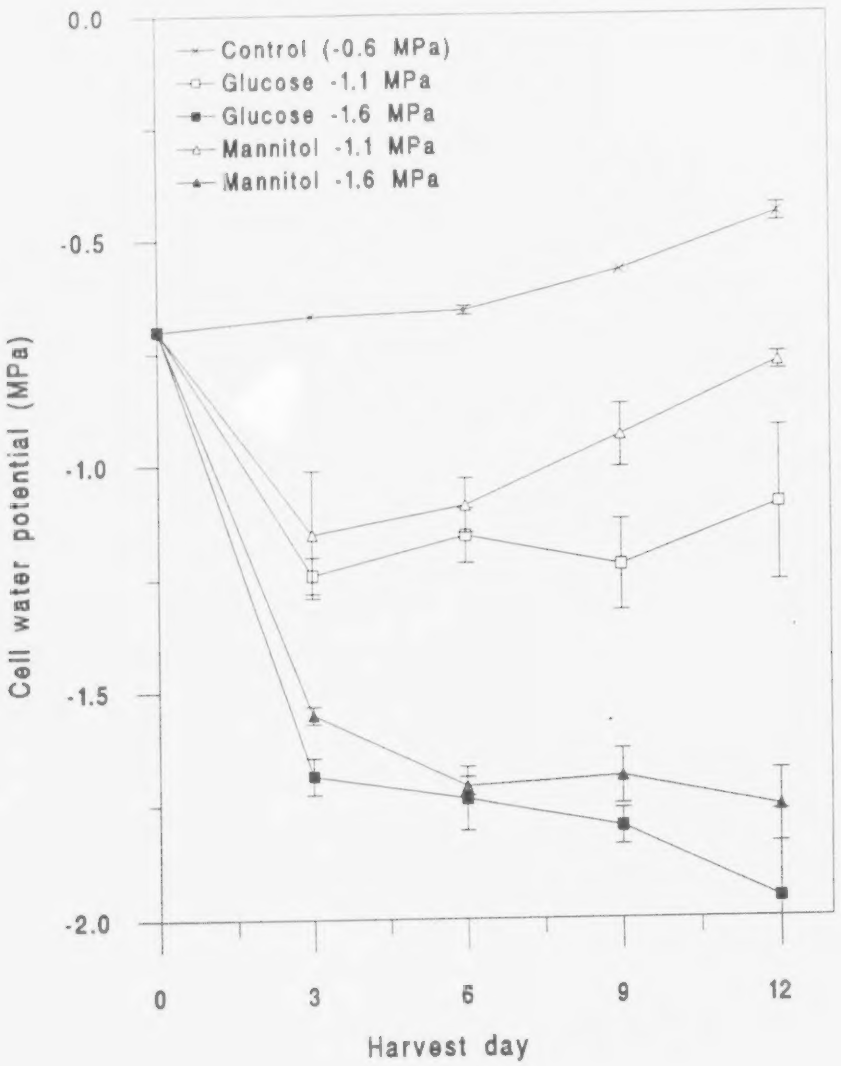

Control (-0.6 MPa)
Glucose -1.1 MPa
Glucose -1.6 MPa
Mannitol -1.1 MPa
Mannitol -1.6 MPa
Cell water potential (MPa)
0.0
-0.5
-1.0
-1.5
-2.0
0
3
6
9
12
Harvest day

Figure III-10. Changes in the cell osmotic potential at different days after culture inoculation in cultures subjected to osmotic stress treatments. Treatments were imposed as described in Figure III-2. Each point is the mean of three replicates. Error bars represent +/- SEM. F test for osmotic stress treatment was significant at $P< 0.01$, for harvest day it was significant at $P<0.05$ and for their interaction it was nonsignificant at $P<0.05$. LSD ($P<0.05$) for mean comparison = 0.27.

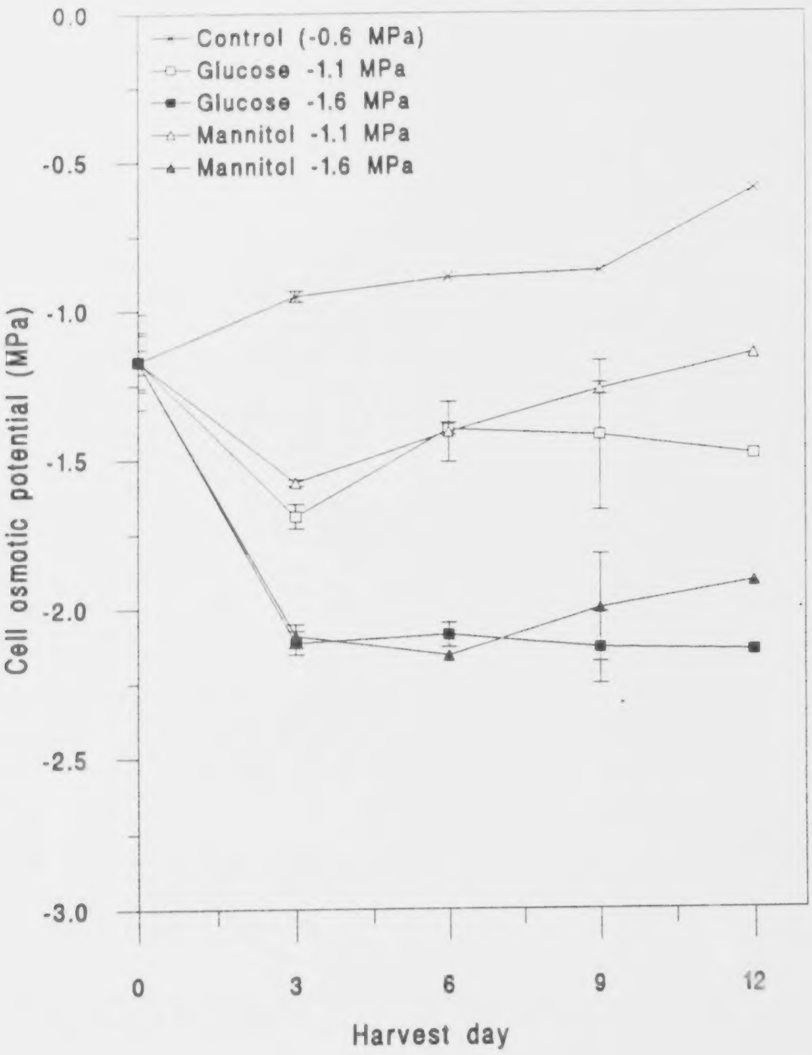

Control (-0.6 MPa)
Glucose -1.1 MPa
Glucose -1.6 MPa
Mannitol -1.1 MPa
Mannitol -1.6 MPa
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
Cell osmotic potential (MPa)
0
3
6
9
12
Harvest day

Figure III-11. Soluble sugar content of the cells at different days after culture inoculation, in cultures subjected to osmotic stress treatments. Treatments were imposed as described in Figure III-2. Each point is the mean of three replicates. Error bars represent +/- SEM. F test for osmotic stress treatments, harvest day and their interactions were significant at $P< 0.01$. LSD ($P<0.05$) for mean comparison = 0.414.

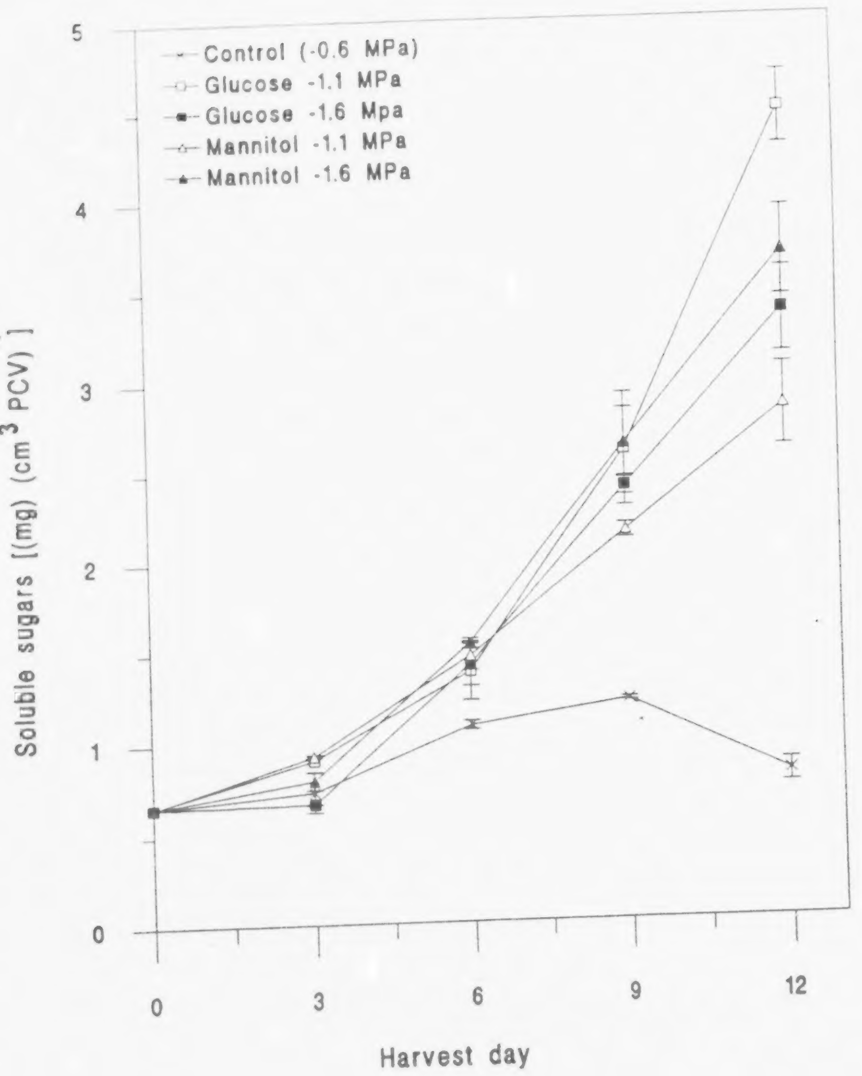

Control (-0.6 MPa)
Glucose -1.1 MPa
Glucose -1.6 Mpa
Mannitol -1.1 MPa
Mannitol -1.6 MPa
Soluble sugars [(mg) (cm ^{3} PCV)$^{-1}$]
0
1
2
3
4
5
0
3
6
9
12
Harvest day

Figure III-12. Free amino acid content per unit PCV at different days after culture inoculation, in cultures subjected to osmotic stress treatments. Treatments were imposed as described in Figure III-2. Each point is the mean of three replicates. Error bars represent +/- SEM. F test for osmotic stress treatments, harvest day and their interactions were significant at $P< 0.01$. LSD ($P<0.05$) for mean comparison = 0.223.

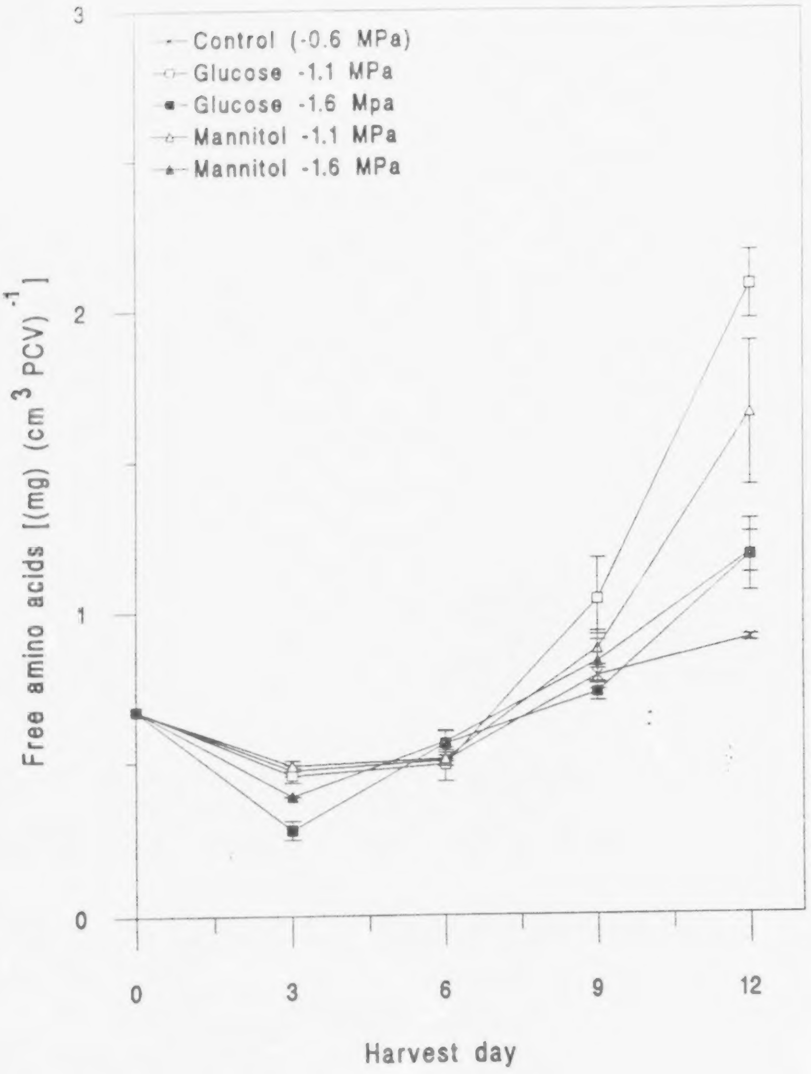

Control (-0.6 MPa)
Glucose -1.1 MPa
Glucose -1.6 Mpa
Mannitol -1.1 MPa
Mannitol -1.6 MPa
Free amino acids [(mg) (cm^3 PCV)$^{-1}$]
0
1
2
3
0
3
6
9
12
Harvest day

Figure III-13. Ratio of soluble sugars to free amino acids at different days after culture inoculation, in cultures subjected to osmotic stress treatments. Treatments were imposed as described in Figure III-2. Each point is the mean of three replicates. Error bars represent +/- SEM. F test for osmotic stress treatments, harvest day and their interactions were significant at $P< 0.01$. LSD ($P<0.05$) for mean comparison = 0.38.

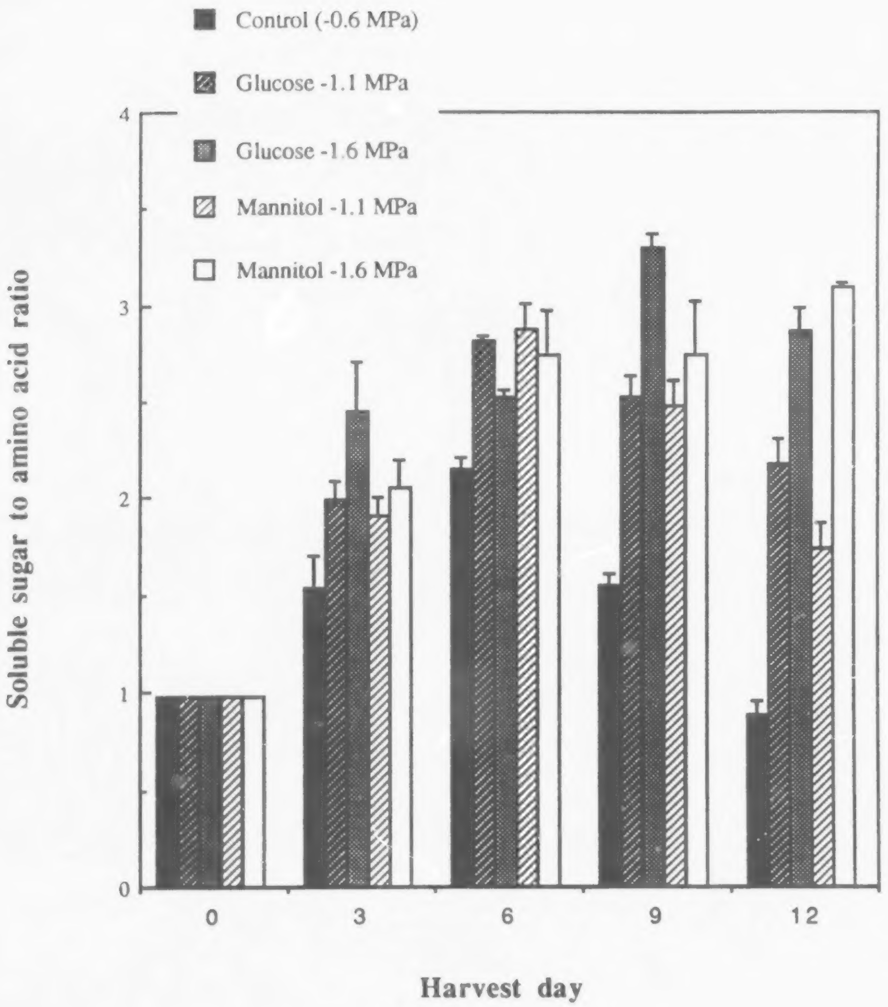
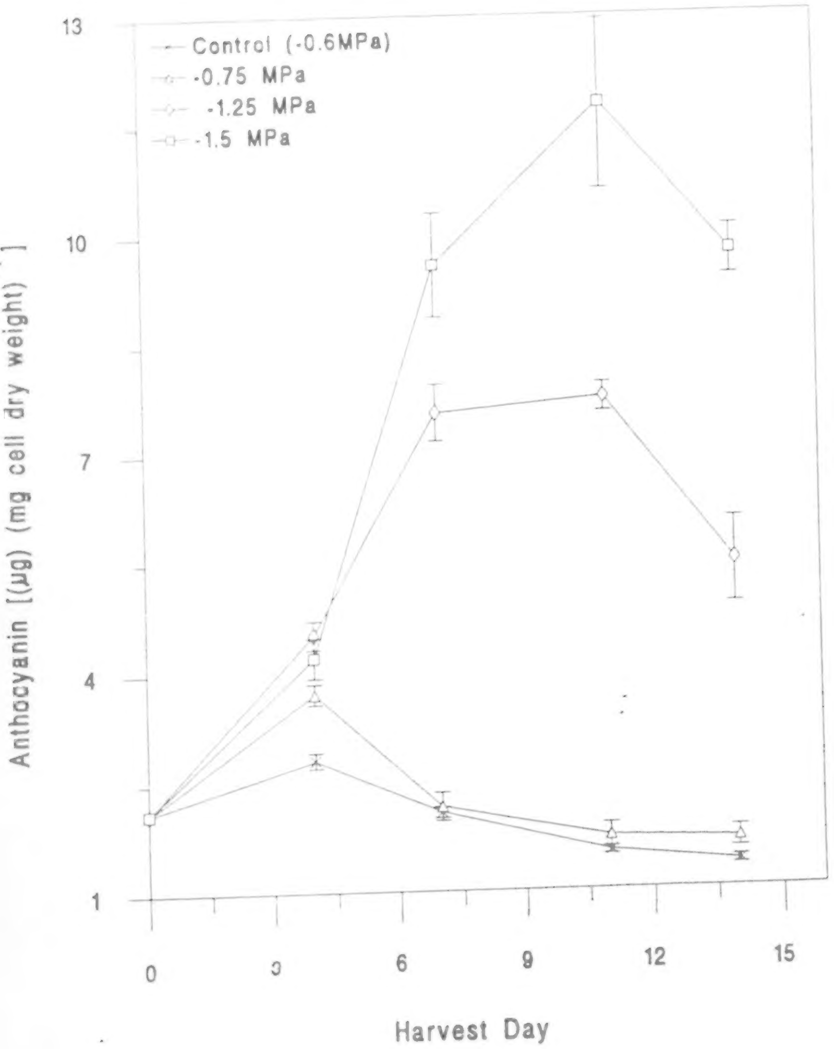

Control (-0.6 MPa)
Glucose -1.1 MPa
Glucose -1.6 MPa
Mannitol -1.1 MPa
Mannitol -1.6 MPa
Soluble sugar to amino acid ratio
0
1
2
3
4
0
3
6
9
12
Harvest day

Figure III-14. Anthocyanin content of the cells at different days after culture inoculation in cultures subjected to osmotic stress with polyethylene glycol (PEG 3350). Cells were subjected to different levels of osmotic stress by inoculating the culture in the medium of low osmotic potentials obtained by the addition of PEG to the MS medium containing 105 mM glucose (medium osmotic potential of -0.6 MPa) which served as the control. Each point is the mean of three replicates. Error bars represent +/- SEM. F test for osmotic stress treatments, harvest day and their interactions were significant at $P< 0.01$. LSD ($P<0.05$) for mean comparison = 1.2.


Control (-0.6MPa)
-0.75 MPa
-1.25 MPa
-1.5 MPa
Anthocyanin [(µg) (mg cell dry weight)$^{-1}$]
13
10
7
4
1
0
3
6
9
12
15
Harvest Day

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Chapter IV

Arylalanine ammonia lyase activity and solute composition of osmotically stressed poplar (*Populus deltoides* var. *occidentalis*) cells in suspension culture in relation to anthocyanin accumulation

IV.1 Introduction

Osmotic stress has been shown to increase the production of various secondary metabolites (DiCosmo & Towers 1984)), including anthocyanins (Do and Cormier 1991). Certain enzymes play an important regulatory role in secondary metabolite production (Behal 1985). The enzyme phenylalanine ammonia lyase (PAL, EC 4.3.1.5) catalyses the elimination of ammonia from L-phenylalanine to give *trans*-cinnamic acid. This reaction is the first step of the phenylpropanoid pathway in plants, which results in the diversion of L-phenylalanine into secondary metabolism. PAL seems to be extraordinarily sensitive to the physiological state of the plant (Jones 1984). PAL activity was shown to be correlated with the synthesis of anthocyanin in a cell suspension culture of a hybrid poplar (Matsumoto et al. 1973). However, PAL activity has not always been correlated with anthocyanin biosynthesis. Constabel et al. (1971) did not find a correlation between PAL activity and the rate of anthocyanin accumulation in cell suspension culture of *Haplopappus gracilis*.

Tyrosine ammonia lyase (TAL, EC 4.3.1) which catalyzes the conversion of L-tyrosine into *para*-coumaric acid has also been reported to play a regulatory role in the biosynthesis of phenolic compounds. While PAL has been found in all examined plants, TAL has been found mainly in monocotyledons (Hanson & Havir 1981) and in a few dicotyledonous species (Nagaoka & Ashihara 1988; Chiang et al. 1987; Beaudoin-Eagan & Thorpe 1985; Gregor 1976).

PAL has been shown to have a relatively broad specificity towards aromatic amino acids, including tyrosine (Hanson & Havir 1981). In fact, a single active site of PAL from maize has been shown to act on both phenylalanine and tyrosine (Havir et al. 1971). Therefore, if TAL activity is detected it would be necessary to determine if it is a separate enzyme or a part of the PAL complex. In cases where both PAL and TAL activities were detected, PAL activity was almost always found to be higher than TAL activity (Hanson & Havir 1981) and therefore, PAL plays a more important role than TAL in the biosynthesis of phenolic compounds.

To explain the magnitude and timing of PAL induction there must be changes in the number of catalytically competent enzyme molecules (Jones 1984). Increased *de novo* synthesis of PAL has been observed when an increase in PAL activity occurred (Wellman & Schopfer 1975; Nakamichi et al. 1983). An increase in the activity of a pre-existing pool of inactive PAL also might contribute to its increased activity (Lau et al. 1980; Jones 1984). In addition to the production of PAL activity, its removal by inactivation or degradation must be postulated to explain the decline in PAL activity observed with time in many tissues (Jones 1984). The loss of PAL activity observed in many tissues may be attributed to the presence of a PAL inactivating system. Zucker (1971) reported that control of PAL activity in cocklebur leaves was primarily through control of the level of a PAL inactivating system.

Osmotic stress results in the accumulation of various solutes. Sugars and amino acids are the major constituents of osmoregulation in many plant species (Fallon & Phillips 1988; Jacomini et al. 1988). Plants also accumulate organic acids in response to osmotic stress (Rudge and Morris 1987).

PAL is a tetramer with only two active sites. Enzymes undergo major conformational adjustments on ligand binding. The changes may include interactions among protomers so that binding on one protomer influences binding at a similar site on another protomer (Hanson & Havir 1981). Different ligands may move the enzymes to binding states that are the same or distinct.

Various amino acids, when supplied through the medium have been shown to increase PAL activity in the yeast *Rhodotorula glutinis* by increasing its *de novo* synthesis. Isoleucine was found to contribute to the stability of PAL activity by inhibiting the synthesis of a PAL inactivating system (Nakamichi et al. 1983). PAL activity has been shown to be regulated by the supply of its substrate phenylalanine in *Phaseolus vulgaris* (Cunha 1987). There must be interactions between the metabolites and enzymes, especially the intermediary metabolites of the shikimic acid, phenylpropanoid and flavanoid pathway on the activity of the regulatory enzymes of secondary metabolism (Hanson & Havir 1981). Gallic acid was found to stimulate PAL activity, while benzoic, caffeic and ferulic acids inhibited PAL activity (Boudet et al. 1971; Alibert et al. 1972). Short term interactions between metabolites and enzymes might provide a fine tuning of the metabolic pathway. *para*-Coumaric acid and *trans*-cinnamic acid were found to inhibit the activity of PAL from barley (Koukol & Conn 1961).

Hence, the solutes that accumulate in the cells in response to osmotic stress might influence PAL activity by influencing its *de novo* synthesis and inactivation or degradation, by direct activation of the enzyme or by increasing the supply of precursors such as phenylalanine.

Studies conducted previously related osmotic stress induced secondary metabolite production with PAL activity. However, there are no reports to relate the solutes accumulated in the cells in response to osmotic stress to PAL activity in cultures accumulating increased amount of secondary metabolites.

I hypothesized that osmotic stress induced increase in anthocyanin production in this cell culture was due to the increase in PAL activity and that the solutes which accumulated in response to osmotic stress, sugars, amino acids and phenolic acids, influence PAL activity.

This Chapter presents the results of studies conducted using cell suspension culture of poplar (*Populus deltoides* var. *occidentalis*) which accumulated increased amount of the anthocyanin pigment cyanidin 3-glucoside when subjected to osmotic stress with mannitol. The objectives of the investigations were to determine i) whether TAL activity was present in this cell culture and if so whether it is a separate enzyme or a part of the PAL complex, ii) whether PAL and TAL played a regulatory role in the biosynthesis of anthocyanin in this cell culture, and iii) whether the organic solutes- soluble carbohydrates, free amino acids and organic acids, (particularly phenolic acids) which accumulate in response to osmotic stress played a role in influencing PAL activity.

IV.2 Materials and Methods

Culture conditions, imposition of osmotic stress treatments and determination of growth and anthocyanin content of the cells are described in Chapter III. Nonstressed cells (Control, medium osmotic potential of -0.6 MPa), and cells stressed with mannitol at a medium osmotic potential of -1.6 MPa, which was found to suppress cell growth and increase anthocyanin accumulation compared with nonstressed cells (Chapter III) were used in the present study.

IV.2.1 Determination of enzyme activities

IV.2.1.1 Extraction of enzymes

Aliquots (15-30 ml depending on the amount of cell needed) of suspension cultured cells from control and mannitol stress treatment were sampled once in three days from the start of the culture inoculation, for up to twelve days for the determination of enzyme activity. The aliquots were filtered through Mira cloth (Calbiochem), the cells were washed twice with 0.1 M sodium borate buffer, pH 8.7, frozen in liquid nitrogen and stored at -80^{0}C for up to one month. All steps in the preparation of the enzyme extract were carried out at 0-4^{0}C. The cells were ground in a mortar and homogenized in 0.1 M sodium borate, pH 8.7 [5 ml. (g fresh weight)$^{-1}$] containing 1 mM dithiothreitol (DTT), 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5% (PVPP) and 0.25 mM fluoride (PMSF). After 30 min, the homogenate was filtered through a Mira cloth. The filtrate was then either passed through a column of activated charcoal or activated charcoal was directly added to the extract to remove anthocyanin. The enzyme extract was centrifuged for 15 min at 40,000x *g*.

IV.2.1.2 Assay of enzyme activities

The clear supernatant was assayed for PAL (Havir 1987) and TAL (Beaudoin-Egan and Thorpe 1985) activities. For the determination of PAL activity, the reaction mixture in 33 mM sodium borate, pH 8.7 consisted of 10 µM L-phenylalanine and 100-200 ul enzyme extract in a total volume of 1.5 ml. TAL activity was assayed using the same buffer and the assay mixture consisted of 5 µM L-tyrosine and 500 ul enzyme extract in a total volume of 1.5 ml. The reaction was initiated by adding the substrate after 10 min preincubation. The reactions of both PAL and TAL were found to be linear for up to 4 hours, under the assay conditions employed. After 2-hour incubation at 30^0C in a water bath, the reaction was stopped by the addition of 50 ul 6 N HCl. After centrifugation at 10,000x *g* for 10 min to remove precipitated proteins, PAL and TAL activities were determined spectrophotometrically by measuring the amount of *trans*-cinnamic acid formed, at 290 nm for PAL and the amount of *para*-coumaric acid formed, at 333 nm for TAL. A standard curve of absorbance vs amount of *t*-cinnamic acid and *p*-coumaric acid in the assay mixture was obtained to convert the absorbance readings to quantities of *t*-cinnamic acid and *p*-coumaric acid formed.

Addition of activated charcoal to the enzyme extract was found to be essential for the complete removal of anthocyanin. Addition of L-aspartic acid, a specific inhibitor of phenylalanine amino transferase activity (Cunha 1987) to the assay mixture was not found to be necessary for the accurate determination of PAL activity in this crude enzyme extract.

The protein content of the extracts was determined after trichloroacetic acid (TCA) precipitation, by the Lowry procedure (Lowry et al. 1951), with bovine serum albumin (BSA) as the standard. The specific activities of the enzymes were expressed as nM of *t*-cinnamic acid (PAL) or *p*-coumaric acid (TAL) formed per mg protein per min.

IV.2.1.3 Identification of the reaction products

The reaction products *t*-cinnamic acid and *p*-coumaric acid were identified by gas chromatography. After the incubation period, the reaction mixture was passed through a 0.5 X 7.0 cm Dowex 50W (200 mesh) anion exchange column (formate form). The column was washed with 10 column volumes of deionized water and the phenolic acids were eluted with 10 column volumes of 1 M formic acid. The eluate was evaporated to dryness in vacuo at 40^0C and trimethylsilyl (TMS) derivatives of phenolic acids were prepared as described by Zwiazek & Shay (1988). The conditions of gas chromatography were as described in Chapter III for soluble carbohydrate analysis, with a different temperature program. Initial oven temperature 60^0C, increased to 205^0C at a rate of 4^0C min^{-1}. Phenolic acids were identified by co-chromatography with *t*-cinnamic acid and *p*-coumaric acid standards.

IV.2.2 Purification and separation of PAL and TAL

Cells from 12-day-old cultures of -1.6 MPa mannitol stress treatment (Chapter III) were used. Twenty five grams fresh weight of cells was washed in 0.1 M sodium borate buffer, pH 8.7 and frozen in liquid nitrogen. The extraction of enzymes was carried out as described in the preceding section (Extraction of enzymes). The enzyme extract was subjected to 65% saturation with ammonium sulfate and the precipitated protein was recovered by centrifugation at 40,000x *g* for 10 min. After dissolving the protein in extraction buffer, solid ammonium sulphate was added to give 30% saturation. The precipitated protein was discarded after centrifugation. The 30-65% fraction was then recovered by 65% ammonium sulphate precipitation and centrifugation as above. The protein was dissolved in 10 ml 0.05 M potassium phosphate buffer, pH 7.0 and subjected to further purification by cation exchange chromatography (carboxymethyl cellulose) and by gel filtration (Sephadex G-200) as described by Kindl (1970). EDTA (2mM), PMSF (0.25 mM) and DTT (1 mM) were included at all stages of purification.

PAL and TAL were separated based on their molecular weight by gel filtration (Sephadex G-200). The protein content of the fractions was determined by measuring the absorbance at 280 nm (Dunn 1989). PAL and TAL activities were determined in the fractions containing protein, as described earlier.

IV.2.3 Quantification of PAL

The amount of PAL in 12-day-old cultures of control and -1.6 MPa mannitol stress treatment was determined by indirect Enzyme-Linked Immunosorbent Assay (ELISA) (competition assay) as described by Harlow and Lane (1988). PAL from the cells was extracted and the protein precipitated between 30-65% ammonium sulphate saturation was collected as described in the preceding section. The precipitate was dissolved in phosphate-buffered saline (PBS) and subjected to ultrafiltration, using Centricon-100 ultrafilters with a molecular weight cut-off of 100,000 and the retentate was recovered. PAL purified by the method described in the preceding section was used for binding to the microtiter wells (Immulon 4, rigid polystyrene, flat bottom plates). Polyclonal antibody to PAL elicited in rabbit (Whetten & Sederoff, 1992) was used as the primary antibody and alkaline phosphatase-coupled goat anti-rabbit immunoglobulin serum was used as the secondary antibody. Colorimetric determination of bound alkaline phosphatase was done using standard methods (Harlow & Lane 1988). The coating enzyme was diluted in PBS, and partially purified PAL obtained from the samples, standard PAL, primary antibody and secondary antibody were diluted in 0.05% BSA/PBS Tween (PBST) containing 0.02%

sodium azide. PAL in the test solution (samples) was quantified by obtaining a standard curve using known amounts of pure PAL and by using the absorbance values of the test solutions which fall within the linear portion of the standard curve for computation of the quantity of PAL.

The protein content of the extracts was determined by the dye binding method (Bradford 1976). PAL quantity in the partially purified sample extract, when expressed on protein basis was calculated based on the total protein present in the crude extract.

IV.2.4 Determination of soluble carbohydrate, free amino acid and organic acid composition of cells

The soluble carbohydrate composition of the cells was determined for the control and stressed cells once every 3 days, from the start of culture inoculation, for up to 12 days. The soluble carbohydrate composition of the cells was determined as described in Chapter III. Free amino acids and organic acids were extracted from the cells as described for the soluble carbohydrates. Amino acids and organic acids were separated by ion exchange chromatography and eluted as described by Zwiazek & Shay (1988). The eluent containing free amino acids or organic acids was evaporated to dryness in vacuo at 30^0C and dissolved in deionized water.

The amino acid composition of control and mannitol stressed cells was determined for days 0, 1, 3, 6, 9 and 12. Amino acids were analyzed as o-phthaldialdehyde derivatives (Jones & Gilligan, 1983). Separation of amino acids was accomplished with the use of a Varian 5000 high performance liquid chromatograph and a Varian Fluorichrom detector (excitation 340 nm, emission 450 nm). Samples were mixed 1:1 with the fluoraldehyde reagent and injected onto a Supelcosil 3 micron LC-18 reverse phase column (4.6 x 150 mm; Supelco) equipped with a guard column (4.6 x 50 mm) packed with Supelco LC-18 reverse phase packing (20-40 μm). Chromatographic peaks were recorded using a Fisher recorder, and integration was accomplished using a Hewlett

Packard 2645 data system with a Hewlett Packard 18652A A/D converter. Amino acids were identified by co-chromatography with amino acid standards and were quantified by using ethanolamine as the internal standard, which was added to the cell extracts.

The organic acid content of the control and stressed cells was determined once in 3 days for up to 12 days. Organic acids were derivatized and analyzed by gas chromatography as described in the preceding section (Identification of reaction products). Quantification of organic acids was accomplished by using a known amount of benzoic acid as the internal standard, which was added to the cell extracts. Organic acids were identified by co-chromatography with organic acid standards.

IV.2.5 Determination of the effect of solutes on *in vitro* phenylalanine ammonia lyase activity

The enzyme assay conditions were as described in the preceding section (Assay of enzyme activities). The enzyme, purified as described earlier was used. The aromatic amino acids L-tyrosine and L-tryptophan were used in the assay mixture at low concentrations due to their poor solubility. The rest of the solutes were used at concentrations of 0.7, 7.0 and 70.0 mM. The effect of the solutes on *in vitro* phenylalanine ammonia lyase activity was expressed as percentage inhibition or activation, with reference to the control, to which the effector (solute) was not added.

IV.2.6 Cellular compartmentation of soluble carbohydrates

The distribution of soluble carbohydrates in the cytoplasm and vacuole was estimated by determining their amount present in the isolated vacuoles. The cytoplasmic content of these carbohydrates was calculated by substracting the amount present in the vacuole from the total amount present in the cells.

Protoplasts were isolated from 12-day-old cultures of control and mannitol stressed (-1.6 MPa) cells, as described in Chapter III (Protoplast isolation). Vacuoles were isolated from the protoplasts by the polybase induced lysis method (Boudet & Alibert 1987) with the following modifications. Medium B consisted of 25 mM Tris-MES buffer, pH 5.8 and 0.7 M sorbitol. Medium C consisted of 25 mM Tris-MES buffer, pH 8.0 and 0.7 M sorbitol. Vacuoles were collected at the interphase of 5/20% Ficoll after centrifugation at 500x *g* for 30 min at 17^0C.

The carbohydrate composition of the aliquots of protoplasts and of isolated vacuoles was determined and quantified by gas chromatography as described in Chapter III (Determination of sugars and amino acids). The anthocyanin content of the aliquots of protoplasts and vacuoles was measured as described in Chapter III (Quantification of anthocyanin content of cells). The final quantitation of the amount of carbohydrates present in the vacuole was done by correlating their amount with the amount of anthocyanin, as described for the quantitation of sucrose in *Beta vulgaris* vacuoles by Leigh et al. (1979).

IV.2.8 Materials

Polyclonal antibody to PAL elicited in rabbit was courteously supplied by Drs R. W. Whetten and R. R. Sederoff, Department of Forestry, North Carolina State University, Raleigh, North Carolina, USA. Alkaline phosphatase coupled goat anti-rabbit immunoglobulin serum, p-nitrophenol phosphate (PNPP), ion exchange and gel filtration materials for chromatography, cellulase and pectolyase for protoplast isolation, DEAE-Dextran, dextran sulfate and Ficoll-400 for vacuole isolation and, sugar, amino acid and organic acid standards were purchased from Sigma Chemical Co. Immulon 4 microtiter wells were purchased from Dynatech Laboratories. Ultrafilters (MWCO 100,000) were purchased from Amicon Inc.

The data were statistically analyzed by the analysis of variance (ANOVA) procedure and least significant difference (LSD, $P<0.05$) was used for mean comparisons.

IV.3 Results

IV.3.1 Anthocyanin content of cells

Anthocyanin content of the cells increased significantly in response to osmotic stress (Figure IV-1). A significant difference in anthocyanin content between control and stressed cells occurred from day 6 and the difference increased further until day 12, mainly due to the increase in the anthocyanin content of the stressed cells. On day 12, the anthocyanin content of the stressed cells was 219% higher than the initial level while that of the control cells decreased by 69%.

IV.3.2 Effect of osmotic stress on phenylalanine ammonia lyase and tyrosine ammonia lyase activities

Both PAL and TAL activities were detected in the cells. The reaction product of PAL activity was identified to be *trans*-cinnamic acid and that of TAL activity was identified to be *para*-coumaric acid by gas chromatography. The specific activities of PAL and TAL in control and osmotically stressed cells are shown in Figure IV-2. Both PAL and TAL activities increased in osmotically stressed cells. PAL activity in stressed cells decreased until day 3, and increased on day 6 and continued to increase until day 12, while TAL activity increased from the start and continued to increase further until the last harvest day. In controls, the activities of both the enzymes decreased with harvest time. In control cells, by day 9, PAL activity was not detectable and TAL activity was very low [0.014 nmol (mg protein)$^{-1}$ min^{-1}]. On day 3, while no significant difference in the PAL activity between control and stress treatments was observed, a significant difference in TAL activity between these two treatments occurred. By day 12, PAL activity in the stressed cells increased by 151% and TAL activity by 71% over the initial activity level. A similar trend in PAL and TAL activities was observed when their activities were expressed on cell dry weight basis (data not presented).

Change in the ratio of PAL activity to TAL activity is presented in Figure IV-2C While this ratio decreased with harvest time in the control, it increased in stressed cells on day 6 after a decrease on day 3, and remained relatively constant until the last harvest day in the stressed cells. The ratio of PAL to TAL activity increased by 45% on day 9 compared with the initial ratio, in the stressed cells

IV.3.3 Separation of PAL and TAL

Chromatography on sephadex G-200 resulted in the complete separation of PAL and TAL activities (Figure IV-3). The fraction which had TAL activity did not show PAL activity and the fractions having PAL activity did not show TAL activity. TAL activity eluted before PAL activity. After separation, the specific activity of TAL [6.82 nmol p-coumaric acid (mg protein)$^{-1}$ min^{-1}] was found to be 54 times higher than that of the crude extract [0.126 nmol p-coumaric acid (mg protein)$^{-1}$ min^{-1}].

IV.3.4 Amount of PAL

Data on the amount of PAL in control and stressed cells on day 12, determined by Enzyme-Linked Immunosorbent Assay (ELISA), are shown in Table IV-1. When expressed on a protein basis, the amount of PAL was found to be 12% of that in stressed cells compared with the control cells, but when expressed on a dry weight basis, the amount in stressed cells was found to be 81% higher than in control cells. Taking the molecular weight of PAL as 320,000, PAL accounted for 0.047% by weight of total protein in control cells and for 0.006% of total protein in stressed cells.

IV.3.5 Protein content of the cells

The protein content of the stressed cells was significantly higher than that of control cells (Figure IV-4). The protein content of the control cells decreased with harvest time and reached a low level by day 9 and remained so until day 12. The protein content of the stressed cells decreased on day 3 and then increased on day 6 and continued to increase further until day 12. Significant difference in the protein content between control and stressed cells occurred starting from day 6 (2.2 times higher in stressed cells) and then onwards the difference increased further, reaching the maximum on day 12 (13 times higher in stressed cells). By day 12, the protein content of the stressed cells increased by 33% compared with the initial level, while that of control cells decreased by 90%.

IV.3.6 Changes in the amino acid composition of cells

IV.3.6.1 Aromatic amino acids

In general, the phenylalanine content of the stressed cells was lower than that of control cells throughout the harvest time (Figure IV-5A). A significant difference in phenylalanine content between control and stressed cells occurred after one day, largely due to the increase in the phenylalanine content of control cells. No significant difference between the two treatments was observed from day 3 to day 9, but on day 12 a large difference was observed between the two treatments (136% higher in control cells than stressed cells) mainly due to the increase in the phenylalanine content of the control cells.

Tyrosine content of both control and stressed cells decreased from the initial level until day 3 and there was no significant difference between the two treatments until this time (Figure IV-5B). A significant difference in tyrosine content of cells between the two treatments occurred starting from day 6, mainly due to the increase in tyrosine content of stressed cells, and higher tyrosine content of stressed cells was maintained until the last harvest day.

No significant difference in tryptophan content of control and stressed cells occurred until day 9 (Figure IV-5C). The tryptophan content of both control and stressed cells decreased after one day and from day 1 to day 9 its level in cells of both the treatments remained fairly constant and it increased in both the treatments on day 12. However, on day 12, the tryptophan content of control cells was 149% higher than that of stressed cells.

IV.3.6.2 Other amino acids

Changes in the level of nonaromatic amino acids is presented in Table IV-2. The significance level of F test for osmotic stress treatment, harvest day and their interactions for the nonaromatic amino acids is presented in Appendix 1.

Alanine was found to be the major amino acid accumulated in the cells. The alanine content of both the control and stressed cells decreased until day 3. From day 6 onwards its level in stressed cells increased until the last harvest day, while in control cells its level remained fairly constant from day 6 to day 12. Aspartic acid content of both control and stressed cells decreased up to day 3 and its level increased starting from day 9 and continued to increase until day 12 in both control and stress treatments. Although an appreciable difference in the aspartic acid content between the two treatments did not occur, its level was slightly higher in stressed cells than that in control cells throughout the harvest time. No significant difference in isoleucine content between the two treatments occurred until day 9, during which time its level remained fairly constant in both the treatments. From day 9 to day 12, the isoleucine content in cells of both the treatments increased, but it was 177% higher in control cells than in the stressed cells, on day 12. Significant difference in the leucine content between control and stressed cells occurred only on day 12. The leucine content of both the treatments remained fairly constant from day 1 to day 9, after an increase on day 1. On day 12, the leucine content of control cells was about twice that of stressed cells. No appreciable difference in the glycine content of control and stressed cells was observed throughout the harvest time. The glycine content in cells of both the treatments decreased until day 3 and it increased in both the treatments starting from day 9. The methionine content in cells of both the treatments increased one day after the imposition of treatments. Its level decreased until day 6 in control cells and until day 9 in stressed cells, after which it again increased in both the treatments. Maximum difference in methionine content of the cells between the two treatments occurred on day 12 (152% higher in control cells than in stressed cells). The valine content of the stressed cells was higher on day 6 and day 9 compared with the control. But on day 12, it was significantly higher in control cells than in stressed cells.

The cellular content of glutamine, glutamic acid, aspargine, serine and histidine was higher in stressed cells compared with the control, but the cellular content of lysine and threonine was higher in control cells than in stressed cells on the last harvest day. Ornithine and arginine content of the cells did not differ between the two treatments (Table IV-2).

The total amino acid content in cells of both the treatments decreased after the imposition of treatments. While the amino acid content of the stressed cells began to increase starting from day 6, it began to increase starting from day 9 in control cells. However, from day 3 to day 12, the amino acid content of the stressed cells was higher than that of control cells (Table IV-2).

IV.3.7 Organic acid content of cells

The organic acid content of the cells at different harvest days in control and mannitol stressed cells is presented in Table IV-3. The significance level of F test for the same is presented in Appendix 2. Among the phenolic acids, the cells were found to accumulate only shikimic acid in detectable levels. The cells also accumulated other organic acids including ascorbic acid, citric acid and malic acid.

The shikimic acid content of both the control and stressed cells increased on day 3 compared with the initial level. While its level continued to increase until the last harvest day in the stressed cells, in control cells its level decreased on day 6 and continued to decrease further until the last harvest day. On day 12, the shikimic acid content of the stressed cells was 217% higher than in the control cells.

The ascorbic acid content of both control and stressed cells decreased until day 6 from the initial level. In stressed cells, its level increased on day 9 and day 12, but its level decreased in the control cells on day 12. Significantly higher ascorbic acid content in the stressed cells compared with that of control cells occurred on day 9 (120%) and day 12 (231%).

The malic acid content of both control and stressed cells decreased on day 3 from the initial level. In control cells its level decreased until day 6, after which it increased until the last harvest day. In the stressed cells malic acid content began to increase from day 6 and continued to increase until day 12. The malic acid content of stressed cells was higher than that of control cells from day 6 to day 12.

The citric acid content of the stressed cells was slightly higher than that of control cells, throughout the harvest time. The total organic acid content of both control and stressed cells decreased on day 3 from the initial level. After an increase in its level from day 3 to day 6, its level decreased from day 9 and continued to decrease until day 12 in cells of both the treatments. Significantly higher organic acid content was observed in the stressed cells compared with that of control cells on day 9 (95%) and day 12 (476%).

IV.3.8 Soluble carbohydrate composition of cells

The amount of various soluble carbohydrates present in control and mannitol stressed cells at different harvest days is presented in Table IV-4, and the significance level of F test for the same is presented in Appendix 3. Sucrose was found to be the major soluble sugar present in both the control and stressed cells. The sucrose content of the control cells decreased from day 3 itself and reached the minimum by day 12, while that of stressed cells increased starting from day 3 and continued to increase until day 9, after which it decreased. On day 12, the sucrose content of control cells decreased by 97% compared with the initial level. On day 9 the sucrose content of the stressed cells was higher by 146% compared with the initial level. A significant difference in the sucrose content between control and stress treatments occurred throughout the harvest time.

Glucose and fructose were found to accumulate in small amounts compared with sucrose. Higher amounts of both glucose and fructose were present in the stressed cells compared with that of control cells throughout the harvest time, except on day 6, when the difference was nonsignificant.

The inositol content of the control cells began to decrease starting from day 6 and continued to decrease until day 12, while that of the stressed cells increased from day 6 and continued to increase until day 9, after which it decreased. Significantly higher amount of inositol was present in stressed cells compared with the control cells at all harvest times, except on day 3.

Mannitol was present only in mannitol stressed cells. The stressed cells accumulated substantial amounts of mannitol. Its level gradually increased with harvest time, reaching the maximum on day 12 [133 μmol (g dry weight)$^{-1}$].

The total soluble carbohydrate composition of the cells generally followed the same trend as that of sucrose, with significantly higher amounts present in the stressed cells compared with that of control cells at all harvest times.

IV.3.9 Cellular compartmentation of soluble carbohydrates

Results showing the percentage distribution of soluble carbohydrates between the cytoplasm and the vacuole for the control and stressed cells are presented in Table IV-5. Most of the sucrose was present in the cytoplasm in both control (81%) and stressed (95%) cells. All of the glucose, fructose, inositol and mannitol present in both control and stressed cells were found to be present in the cytoplasm.

IV.3.10 Effect of accumulated solutes on *in vitro* PAL activity

Because PAL activity was up to 9 times higher in stressed cells than in control cells, and also because PAL activity increased to greater levels than TAL activity in response to osmotic stress, studies on the effect of various amino acids, organic acids and soluble carbohydrates, whose concentrations were found to vary in response to osmotic stress, on *in vitro* enzyme activity was restricted to PAL (Table IV-6).

Of all the solutes tested only L-tyrosine and L-tryptophan were found to increase *in vitro* PAL activity. L-tyrosine did not affect PAL activity at 0.18 mM but was effective at 0.35 and 3.5 mM and ineffective at 7.0 mM. L-tryptophan activated PAL at 0.09, 0.18 and 0.35 mM but inhibited at 0.7 and 3.5 mM. The maximum PAL activity induction by L-tyrosine was 10% (at 3.5 mM concentration) while that induced by L-tryptophan was 105% (at 0.35 mM concentration).

L-ascorbic acid, L-malic acid and citric acid were all found to be inhibitory to PAL activity. L-ascorbic acid was effective at concentrations of 7.0 and 70.0 mM while L-malic acid and citric acid were effective only at a concentration of 70.0 mM.

The amino acids aspartic acid, alanine, methionine, histidine, isoleucine, leucine, lysine, glycine, serine, threonine, aspargine, glutamic acid and glutamine, the phenolic acid shikimic acid, and the soluble carbohydrates sucrose, glucose, mannitol and inositol were all found to have no effect on *in vitro* PAL activity at the three concentrations tested.

IV.4 Discussion

Both PAL and TAL activities were detected in the cells. It was once thought that only monocotyledons possess TAL activity (Brown and Neish 1955; McCalla & Neish 1959). Subsequently TAL activity has been reported in a few species of dicotyledons (Nagaoka & Ashihara 1988; Beaudoin-Eagan & Thorpe 1985). However, Havir (1987) could not detect TAL activity in soybean tissue culture. The present observation appears to be the first report of TAL activity in poplar.

The activities of both PAL and TAL increased in osmotically stressed cells (Figure

IV-2), which accumulated high anthocyanin levels. It is possible that both PAL and TAL played a regulatory role in osmotically induced anthocyanin production in this cell culture. Such a relationship between anthocyanin biosynthesis and PAL activity has been observed previously in cell suspension culture of a hybrid poplar (Matsumoto et al., 1973). However, Constabel et al (1971) did not find a correlation between PAL activity and anthocyanin accumulation in the cell culture of *Haplopappus gracilis*.

TAL activity, but not PAL activity, increased from the initial level in the stressed cells on the third day. However, in the stressed cells, by the last harvest day the increase in PAL activity was two fold higher than the increase in TAL activity. From this observation it can be implied that TAL activity was more readily induced than PAL activity and hence TAL might play a more important role in the short term regulation of anthocyanin levels and PAL might play a more important role in the long term regulation of the anthocyanin levels in this culture.

The ratio of PAL activity to TAL activity (Figure IV-2C) increased in response to osmotic stress treatment from day 6 to day 12, indicating that PAL activity increases more than TAL activity in response to osmotic stress.

TAL activity was lower than that of PAL in both control and stressed cells throughout the harvest time and the ratio of PAL activity to TAL activity varied from 2.4 to 9.5 in this cell culture. In most cases where both PAL and TAL activities have been reported, PAL activity was always higher than TAL activity (Nagaoka & Ashihara 1988; Hanson & Havir 1981). The present results are in agreement with these reports. On the other hand, Beaudoin-Egan and Thorpe (1985) found higher TAL activity than PAL activity in shoot-forming tobacco callus.

PAL and TAL activities were completely separated by gel filtration (Figure IV-3). The fraction with TAL activity did not show PAL activity and the fractions with PAL activity did not show TAL activity clearly indicating that PAL and TAL are two different enzymes. These results are in agreement with the findings of Beaudoin-Egan & Thorpe

(1985) who found that PAL and TAL from tobacco callus are two different enzymes. Our findings are contrary to the previous observations that PAL itself shows TAL activity (Hanson & Havir 1981; Havir et al. 1971).

TAL eluted earlier than PAL from the sephadex column indicating that it has higher molecular weight than PAL. These results are in agreement with the previous findings (Kindl 1970; Beaudoin-Eagan & Thorpe 1985).

Because TAL activity was much lower than PAL activity, studies on the quantitation of the enzyme and the possible role of accumulated solutes on enzyme activity were restricted to PAL. To explain the large variation in the enzyme activity between the treatments and with time, there must be changes in the number of catalytically competent enzyme molecules rather than large variations in the catalytic efficiency of a fixed amount of already existing enzyme (Jones 1984). Determination of the amount of PAL (Table IV-1) showed that it was higher in stressed cells than in the control cells on day 12 when expressed on a cell dry weight basis, indicating that there was a higher amount of PAL protein in the stressed cells and this might be responsible for the higher PAL activity of the stressed cells. However, the amount of PAL was lower in stressed cells when expressed on a protein basis. This is likely due to the greater increase in the amount of other proteins compared with PAL protein.

The total protein content of the stressed cells was also higher than the control cells (Figure IV-4). While the protein content of the stressed cells increased with harvest time, it decreased with harvest time in control cells. A similarity in the trend of change in protein content (Figure IV- 4) and PAL activity (Figure IV-2A) of control and stressed cells at different harvest times was observed, indicating that an increase in PAL protein might be responsible for the higher PAL activity of the stressed cells and decrease in PAL protein might be responsible for the lower PAL activity of control cells.

On the last harvest day even though a small amount of PAL protein was detected in the control cells, PAL activity was completely absent. This suggests that the decreased

PAL activity with harvest time in control cells may not only be due to the degradation of PAL protein but probably also due to its inactivation. The polyclonal antibody to PAL that was used for the quantitation of PAL by ELISA was perhaps able to bind to the completely inactive form of PAL. However, the possibility of some nonspecific binding of the antibody cannot be ruled out.

These observations suggest that an increase in PAL synthesis and a decrease in degradation and/or inactivation of PAL might be responsible for the higher PAL activity of the stressed cells. Previous studies have shown that an increased synthesis of PAL was responsible for the higher PAL activity (Nakamichi et al. 1983; Wellman & Schopfer 1975) and increased level of a PAL inactivating system was responsible for the decrease in PAL activity (Zucker 1971).

The observation that PAL accounted for 0.047% by weight of total protein in control cells and for 0.006% in stressed cells is in agreement with the previous findings that less than 0.1% of total cellular protein can be accounted for by PAL (Jones 1984).

The observation that the protein content of the stressed cells increased with harvest time and was higher than that of control cells indicated that protein synthesis was higher in stressed cells, which showed higher PAL activity and anthocyanin content. These observations are in contrast with the previous findings that conditions unfavorable for protein synthesis in plants result in the increased production of phenolic compounds (Gershenzon 1984).

An increase in the activity of the enzyme could also result from the activation of an existing pool of enzyme, in addition to its *de novo* synthesis and the inhibition of inactivation and/or degradation. The solutes accumulated in the cells when subjected to osmotic stress might influence PAL activity by influencing any of the above processes. Osmotic stress was found to increase the cellular content of various amino acids (Figure IV-5 and Table IV-2), organic acids (Table IV-3) and soluble carbohydrates (Table IV-4) in this cell culture. Such increase in the level of amino acids (Gershenzon 1984), organic acids (Morgan 1984) and soluble carbohydrates (Fallon and Phillips, 1989) when plants are subjected to osmotic or water stress is well known.

PAL is a cytoplasmic enzyme (Hanson & Havir 1981). Determination of the soluble carbohydrate composition of isolated vacuoles showed that most of the sucrose and all of glucose, fructose, inositol and mannitol accumulated in the cytoplasm in both control and stressed cells (Table IV-5). Amino acids have been known to accumulate in the cytoplasm (Borowitzka 1981). Although organic acids are found to be accumulated mainly in the vacuole, they are likely to be present at a low concentration in the cytoplasm also (Salisbury & Ross 1985). Thus all those organic solutes whose levels vary in response to osmotic stress might influence PAL activity.

Changes in the phenylalanine content with harvest time in control and stressed cells (Figure IV-5A) indicated that there was no correlation between phenylalanine level and PAL activity. The increase in the phenylalanine level of control cells (day 12) subsequent to the absence of PAL activity (day 9), indicated that limitation of phenylalanine supply was not responsible for the decrease in PAL activity of control cells. This increase in phenylalanine content of control cells on the last harvest day might be due to its non-utilization by PAL and consequent non-utilization for anthocyanin biosynthesis, resulting in its accumulation. These results are contrary to the observations made by Cunha (1987) in *Phaseolus vulgaris* that PAL activity is regulated by phenylalanine supply and availability. The increase in the phenylalanine level subsequent to the absence of PAL activity as found in this culture has been observed in tobacco cell suspension cultures subsequent to inhibition of PAL activity by 2-aminooxy-3-phenylpropionic acid (Hanson & Havir 1981).

The tyrosine content of the stressed cells was higher than that of control cells through out the harvest time (Figure IV-5B). L-tyrosine at concentrations of 0.35 and 3.5 mM stimulated PAL activity *in vitro* while at 0.18 mM concentration it was ineffective (Table IV-6). Therefore, it is likely that *in vivo* tyrosine was present in stressed cells at

PAL activating concentration while it was at an ineffective concentration (lower) in control cells. Tyrosine was ineffective at the high concentration tested (7 mM). If tyrosine acted as a competitive effector of PAL activity, due to the presence of a high amount of other solutes, the tyrosine concentration present in the stressed cells might not have been inhibitory to PAL activity *in vivo*. Koukul and Conn (1961) however, found L-tyrosine to be inhibitory to the activity of PAL from barley.

There was no significant difference in the tryptophan content of control and stressed cells until day 12, when it was higher in control cells than in stressed cells (Figure IV-5C). *In vitro*, tryptophan stimulated PAL activity at very low concentrations (0.09, 0.18 and 0.35 mM) and inhibited PAL activity at concentrations of 0.7 and 3.5 mM. If the effect of tryptophan on PAL activity is influenced by the presence of other solutes in the cell (competitive effect), because stressed cells accumulated higher amounts of osmotic solutes, it is likely that at the same amount of tryptophan present in both control and stressed cells, tryptophan could have stimulated PAL activity in stressed cells and inhibited PAL in control cells. Behal (1985) observed that PAL activity was increased in the basidiomycete *Oudemansiella mucida* by tryptophan supplied through the medium. The large increase in tryptophan content of control cells observed on the last harvest day might not have had any effect on inhibiting PAL activity, because PAL activity was completely absent by the 9th day itself, when there was no significant difference in the tryptophan content between control and stressed cells.

L-tryptophan was more effective than L-tyrosine in affecting PAL activity because the maximum increase in PAL activity induced by tryptophan was 105%, while it was only 10% by tyrosine. PAL activity was also more sensitive to tryptophan than tyrosine because the minimum activating concentration of tyrosine was 0.35 mM while that of tryptophan was 0.09 mM.

The effective concentration of both tyrosine and tryptophan on *in vitro* PAL activity are low. Because of their poor solubility they are likely to be present at low concentrations in the cells. Hence the concentrations tried *in vitro* might be within the range of their *in vivo* concentrations.

The activation of PAL at low concentrations and inhibition of or ineffectiveness on PAL activity at high concentrations of tyrosine and tryptophan could be similar to the negative co-operativity shown by gallic acid with respect to its own binding to PAL. Hanson & Havir (1981) explained the increased PAL activity brought about by gallic acid (Boudet et al 1971; Alibert et al 1972) at low concentration to be due to its binding to the first site and the decreased PAL activity at high concentration to be due to the restoration of symmetry on binding to the second site.

The observed increase in PAL activity by tyrosine and tryptophan was not due to their utilization as substrates because there was no increase in PAL activity (absorbance @ 290 nm) in the absence of phenylalanine in the assay mixture. Similar results on the effect of tyrosine and tryptophan on *in vitro* PAL activity were obtained when crude enzyme was used. Hanson & Havir (1981) stated that the enzyme is subjected to changes in properties during purification. The similarity between the results obtained with both crude and purified enzyme further confirmed the effects of tyrosine and tryptophan on *in vitro* PAL activity.

The cellular content of phenylalanine increased a day after the imposition of treatments while that of tyrosine and tryptophan decreased in both control and stressed cells. Such an increase in the level of phenylalanine and decrease in the level of tyrosine immediately after transfer of cells to fresh medium was observed by Nagaoka and Ashihara (1988). However, the significance of this is not known.

Although changes in the cellular content of several non-aromatic amino acids occurred in response to osmotic stress, (Table IV-2) none of them had an effect on *in vitro* PAL activity, indicating that these non-aromatic amino acids might not have played a role in influencing the activity of the existing pool of PAL in the cells. These results on *in vitro* PAL activity are in agreement with the findings of Parkhurst & Hodgins (1972) that alanine, isoleucine, leucine and valine had no effect on the activity of yeast PAL *in vitro* and in contrary to their findings that glycine was inhibitory to PAL activity.

Among the phenolic acids only shikimic acid was found to accumulate in small quantities (Table IV-3). Although its level was higher in the stressed cells than in the control cells in the later harvest days (day 9 to 12), it did not have any effect on *in vitro* PAL activity up to 100 mM concentration. The cells did not accumulate the phenolic acids cinnamic acid, coumaric acid (Koukul & Conn, 1961), gallic acid, benzoic acid, ferulic acid or salicylic acid (Hanson & Havir 1981) which are all known to influence PAL activity, at detectable levels. This indicates that they might not have played a role in influencing PAL activity in this culture. A similar observation was made by Whetten & Sederoff (1992) who reported that none of the intermediates of the phenylpropanoid pathway accumulated to significant levels in the differentiating xylem of lodgepole pine.

The cellular content of ascorbic acid, malic acid and citric acid was found to be higher in stressed cells (Table IV-3) but they inhibited *in vitro* PAL activity at a concentration of 7 mM or higher (Table IV-6). However, because organic acids are known to accumulate mostly in the vacuole and also because high PAL activity was observed in stressed cells, these organic acids might not have accumulated in the cytoplasm at concentrations that are inhibitory to PAL activity.

The cellular content of the soluble carbohydrates sucrose, glucose, fructose, inositol and mannitol was higher in stressed cells (Table IV-4). But none of them had effect on *in vitro* PAL activity, indicating no role for them in influencing the activity of the existing pool of PAL. The ineffectiveness of many of these solutes, especially of the major sugar (sucrose) and the major amino acid (alanine) accumulated in the cells, at different concentrations on *in vitro* PAL activity indicates that change in the osmotic potential of the stressed cells might not have influenced the activity of the existing pool of PAL *in vivo*.

Although many of the accumulated solutes did not seem to play a role in influencing the activity of the existing pool of PAL, some of these solutes may have influenced PAL activity in these cells by influencing its *de novo* synthesis and/or, synthesis or activation of the PAL inactivating .system. Nakamichi et al. (1983) observed an increase in PAL activity due to its *de novo* synthesis in the yeast *Rhodotorula glutinis* when tyrosine, tryptophan, phenylalanine, leucine, valine or methionine were supplied through the medium. Their findings also showed that isoleucine stabilized PAL activity by inhibiting the action of the PAL inactivating system or by repressing the synthesis of the PAL inactivating system.

Aspartic acid might influence PAL activity by making phenylalanine available by inhibiting the activity of phenylalanine amino transferase (Cunha 1987) which diverts phenylalanine to protein synthesis. However, lack of correlation among aspartic acid level, phenylalanine level and PAL activity indicated that aspartic acid might not have played any role in influencing PAL activity in this cell culture.

Havir (1979) reported that a carbohydrate is associated with PAL. If this post-translational modification is important for PAL activity, the presence of a higher amount of soluble carbohydrates in the stressed cells might have played a role in increasing the PAL activity of the stressed cells. The prosthetic group of PAL might contain a dehydroalanine residue and the prosthetic group of the above type could be formed by the modification of normal amino acids such as serine, cysteine and their derivatives (Hanson & Havir 1981). Higher amino acid content of the stressed cells might have favored the formation of the prosthetic group and hence might have contributed to increased PAL activity.

From the results of the present study it can be concluded that both PAL and TAL play a regulatory role in anthocyanin biosynthesis of suspension cultured cells of poplar. The solutes accumulated in response to osmotic stress might have increased PAL activity by influencing the activity of the existing pool of PAL and also by influencing its synthesis, inactivation and/or degradation. The PAL activity changes that occurred in the cells are a result of the interplay of all these processes. While *de novo* synthesis of PAL or PAL inactivating system might provide a means for the long term and coarse regulation of PAL activity, direct activation or inactivation of PAL or PAL inactivating system by accumulated solutes might provide a means for the short term and fine regulation of PAL activity. Further investigations on the effect of osmotic stress on *de novo* synthesis of PAL or PAL inactivating system at the transcription, translation and post translation level is needed to understand the mechanism of osmotic stress induced increase of PAL activity. Such investigations should help in determining the rate limiting step in the appearance of enzyme activity in response to a stimulus, at the molecular level.

Table IV-1. Amount of PAL protein in control and mannitol-stressed cells 12 days after culture initiation. Treatments were imposed as described in Figure IV-1. The phenylalanine ammonia lyase (PAL) protein content was determined by indirect ELISA (competition assay), after partial purification of the enzyme. The PAL protein content is expressed on total protein basis. Values are means of three replicates. Figures in parenthesis are +/- standard error.

Treatment	Quantity of PAL	
	nmol (mg Protein)$^{-1}$	pmol (mg dry wt)$^{-1}$
Control	1.48 (0.06)	2.18 (0.09)
Stress	0.18 (0.01)	3.94 (0.12)

Table IV-2 Amount [μmol (g dry weight)$^{-1}$] of various free amino acids in control and mannitol stressed cells at different days after culture inoculation. Treatments were imposed as described in Figure IV-1. Values are means of three replicates. Figures in parenthesis are +/- standard error. Least significant difference (LSD) values at 0.05 level of significance for mean comparison are presented

Treatment		Control					Stress					LSD
Harvest day	0	1	3	6	9	12	1	3	6	9	12	
Alanine	213	109 (2.8)	45 (2.2)	24 (1.4)	30 (1.1)	23 (1.0)	113 (0.9)	72 (0.7)	104 (25.5)	114 (10.7)	198 (18.4)	31.27
Aspartic acid	11.0	4.3 (0.15)	0.9 (0.07)	0.6 (0.01)	2.8 (0.11)	9.8 (1.01)	4.9 (0.28)	1.7 (0.56)	0.9 (0.21)	5.1 (0.83)	10.7 (1.35)	1.80
Isoleucine	5.2	4.3 (0.06)	3.5 (0.13)	3.1 (0.10)	4.4 (0.31)	25.8 (0.52)	2.8 (0.05)	3.1 (0.09)	3.9 (0.70)	4.1 (0.3)	9.3 (2.35)	2.38
Leucine	1.5	6.9 (0.21)	5.4 (0.21)	5.6 (0.19)	6.8 (0.48)	24.1 (0.75)	4.8 (0.09)	4.7 (0.06)	5.8 (1.04)	5.9 (0.39)	11.7 (2.42)	2.64
Glycine	15.9	3.0 (1.06)	1.4 (0.24)	1.3 (0.07)	4.7 (0.06)	9.8 (0.09)	3.5 (1.36)	1.9 (0.04)	3.7 (5.90)	5.4 (5.07)	8.9 (9.13)	1.87
Methionine	0.5	1.4 (0.13)	0.8 (0.02)	0.4 (0.14)	0.8 (0.07)	1.7 (0.09)	1.1 (0.02)	0.9 (0.01)	0.8 (0.10)	0.5 (0.03)	0.7 (0.04)	0.24
Valine	9.0	10.2 (0.02)	6.5 (0.29)	4.4 (0.18)	6.6 (0.30)	50.3 (1.37)	8.2 (0.16)	8.8 (0.20)	12.9 (2.61)	16.3 (0.19)	34.8 (6.56)	6.73
Glutamine	29.6	17.7 (1.06)	4.3 (0.24)	1.6 (0.07)	2.9 (0.06)	1.3 (0.09)	23.7 (1.36)	24.3 (0.04)	25.2 (5.90)	61.3 (5.07)	72.9 (9.13)	11.31

Contd.

...Contd

Table IV-2.

Treatment		Control					Stress					LSD
Harvest day	0	1	3	6	9	12	1	3	6	9	12	
Glutamic acid	6.3	30.4 (1.43)	7.2 (0.34)	1.9 (0.14)	6.5 (0.82)	13.7 (0.26)	14.3 (0.47)	9.2 (0.84)	8.6 (2.00)	12.9 (1.06)	26.1 (4.23)	4.83
Aspargine	28.8	5.4 (0.11)	5.9 (0.57)	1.9 (0.23)	4.2 (0.35)	17.7 (0.94)	4.6 (0.10)	7.2 (0.46)	7.3 (1.62)	14.3 (0.56)	46.2 (5.53)	5.53
Serine	19.4	13.6 (2.54)	4.7 (0.38)	1.8 (0.08)	6.6 (0.25)	31.0 (1.37)	10.2 (0.26)	7.7 (0.07)	15.2 (5.13)	22.4 (2.23)	56.0 (11.02)	11.85
Histidine	6.5	5.9 (0.32)	2.3 (0.16)	0.9 (0.04)	2.3 (0.29)	9.0 (0.31)	6.1 (0.09)	3.8 (0.18)	4.6 (1.14)	7.0 (0.31)	11.2 (0.74)	1.41
Lysine	12.7	0.8 (0.07)	1.4 (0.21)	1.4 (0.09)	2.2 (0.40)	11.4 (0.62)	0.8 (0.03)	1.3 (0.19)	1.9 (0.46)	3.1 (0.32)	5.9 (1.26)	1.49
Threonine	9.6	6.5 (0.17)	3.2 (0.21)	2.3 (0.13)	6.4 (0.35)	31.4 (1.34)	5.9 (0.09)	4.4 (0.04)	6.5 (1.64)	9.3 (0.88)	21.3 (4.45)	4.69
Ornithine	7.2	3.1 (0.78)	2.9 (0.07)	2.6 (0.08)	2.8 (0.10)	2.8 (0.02)	3.0 (0.03)	2.9 (0.09)	2.4 (0.13)	2.8 (0.03)	2.6 (0.12)	0.24
Arginine	2.5	21.8 (0.58)	5.2 (0.35)	2.0 (0.11)	7.5 (0.50)	13.7 (0.78)	19.1 (0.43)	7.6 (0.17)	4.4 (0.92)	6.8 (0.75)	11.0 (0.38)	2.05
Total	593	376 (4.2)	144 (6.4)	93 (3.9)	136 (4.3)	333 (10.7)	342 (4.6)	213 (2.8)	288 (65.4)	355 (26.2)	601 (53.8)	83.94

Table IV-3. Amount [μmol (g dry weight)$^{-1}$] of various organic acids in control and mannitol-stressed cells at different days after culture inoculation. Treatments were imposed as described in Figure IV-1. Values are means of three replicates. Figures in parenthesis are +/- standard error. Least significant difference (LSD) values at 0.05 level of significance for mean comparison are presented.

Treatment		Control				Stress				LSD ($P<0.05$)
Harvest day	0	3	6	9	12	3	6	9	12	
Shikimic acid	0.24	0.61 (0.13)	0.53 (0.10)	0.37 (0.07)	0.22 (0.05)	0.39 (0.00)	0.42 (0.16)	0.60 (0.22)	0.68 (0.08)	0.31
Ascorbic acid	5.91	4.64 (0.27)	2.53 (0.54)	2.83 (0.74)	0.27 (0.26)	4.38 (0.66)	2.39 (0.38)	6.23 (0.71)	6.54 (0.72)	1.80
Malic acid	12.44	2.18 (0.64)	1.05 (0.10)	1.41 (0.51)	2.01 (0.51)	1.51 (0.45)	1.73 (0.44)	3.05 (0.68)	3.95 (0.89)	1.70
Citric acid	4.29	3.95 (0.75)	9.49 (3.22)	3.51 (1.08)	0.38 (0.06)	6.67 (1.06)	14.99 (4.96)	5.87 (1.42)	3.61 (0.61)	4.89
Total	22.64	10.77 (0.70)	16.07 (0.43)	7.75 (0.45)	2.37 (1.09)	12.56 (1.06)	19.46 (4.84)	15.14 (0.91)	13.62 (1.83)	4.37

Table IV-4. Amount [µmol (g dry weight)$^{-1}$] of various soluble carbohydrates in control and mannitol-stressed cells at different days after culture inoculation. Treatments were imposed as described in Figure IV-1. Values are means of three replicates. Figures in parenthesis are +/- standard error. Least significant difference (LSD) values at 0.05 level of significance for mean comparison are presented.

Treatment		Control				Stress				LSD (P<0.05)
Harvest day	0	3	6	9	12	3	6	9	12	
Sucrose	73.4	50.1 (1.29)	31.9 (0.81)	32.2 (3.58)	2.2 (0.39)	122.6 (3.71)	161.8 (4.45)	180.6 (11.63)	98.9 (15.14)	21.6
Glucose	8.9	6.3 (0.91)	13.7 (1.64)	6.0 (0.92)	0.9 (0.18)	15.2 (0.45)	12.0 (0.21)	17.3 (3.83)	6.2 (1.38)	5.4
Fructose	22.5	6.0 (0.27)	12.4 (0.72)	4.9 (0.23)	2.3 (0.06)	10.6 (1.50)	10.7 (0.69)	11.0 (1.34)	8.2 (0.51)	3.0
Inositol	15.0	16.4 (0.35)	14.1 (0.92)	11.6 (0.90)	0.4 (0.22)	14.8 (1.38)	27.4 (0.30)	33.3 (1.06)	21.0 (1.42)	2.8
Mannitol	0.0	0.0	0.0	0.0	0.0	76.4 (2.77)	107.5 (1.47)	132.7 (12.69)	93.1 (9.81)	17.3
Total	119.7	78.7 (0.69)	72.1 (1.64)	54.6 (5.06)	5.9 (0.36)	219.0 (5.14)	288.3 (4.00)	349.7 (3.28)	266.9 (8.02)	12.8

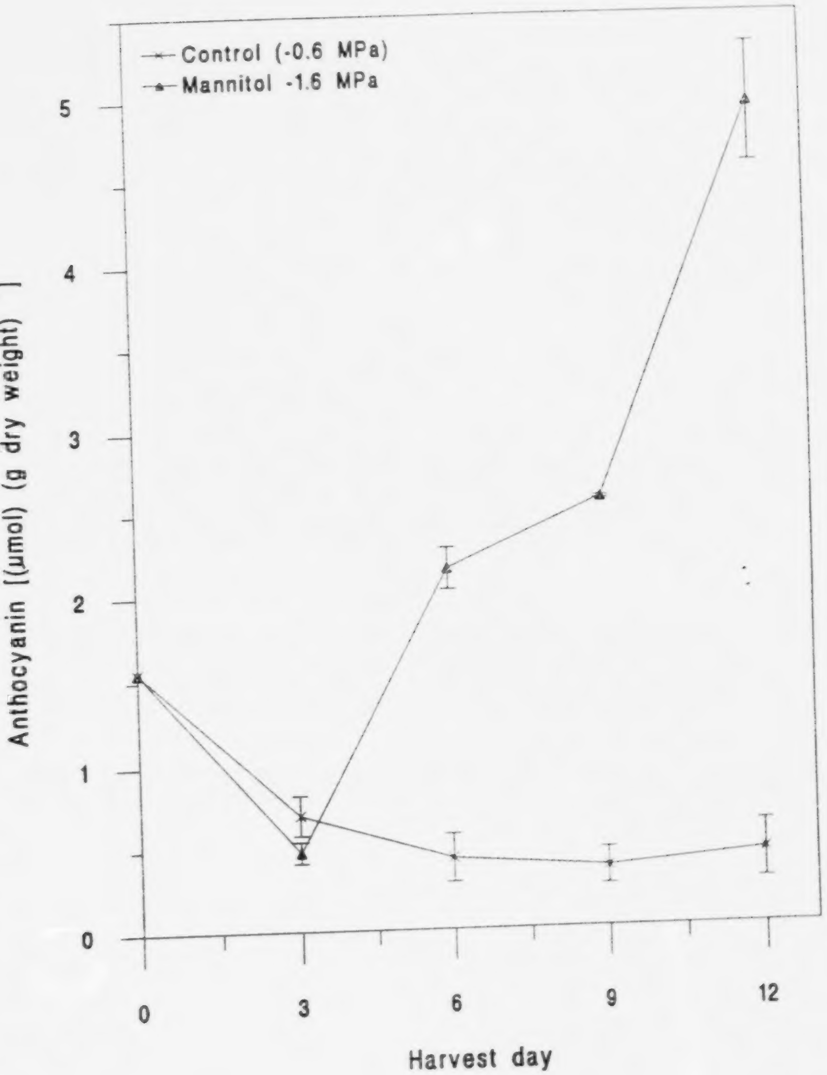
Table IV-5. Amount of soluble carbohydrates in the vacuole and cytoplsm in control and mannitol stressed cells 12 days after culture initiation. Treatments were imposed as described in Fig. 1. The vacuolar content of soluble carbohydrates was determined using isolated vacuoles and the cytoplasmic content was calculated by substracting the amount present in the vacuole from the total amount present in the cells. Values are means of three replicates. Values in parenthesis indicate +/- standard error.

Soluble carbohydrate	Control			Stress		
	Amount in vacuole [µmol (g dry wt.)$^{-1}$]	Amount in cytoplasm [µmol (g dry wt.)$^{-1}$]	% in cytoplasm	Amount in vacuole [µmol (g dry wt.)$^{-1}$	Amount in cytoplasm [µmol (g dry wt.)$^{-1}$	% in cytoplasm
Sucrose	1.47 (0.16)	6.25 (0.82)	81	1.17 (0.06)	21.47 (3.41)	95
Glucose	0.0	2.10 (0.08)	100	0.00	1.82 (0.06)	100
Fructose	0.0	3.90 (0.60)	100	0.00	2.41 (0.38)	100
Inositol	0.0	1.02 (0.11))	100	0.00	5.20 (0.26)	100
Mannitol	0.0	0.00 (0.00)	100	0.00	22.5 (1.66)	100

Table IV-6. Effect of various solutes on *in vitro* phenylalanine ammonia lyase (PAL) activity. Percentage inhibition or activation is expressed with reference to the control, to which the effector solute was not added. Values in parenthesis represent +/- standard error.

Compound	Conc. (mM)	PAL activity (nmol t-cinnamic acid sample^{-1} h^{-1})	Effect
None (Control)	------	5.50 (0.00)	-------------------
L-Tyrosine	0.18	5.50 (0.00)	No effect
	0.35	6.00 (0.02)	9% Activation
	3.50	6.05 (0.04)	10% Activation
	7.00	5.50 (0.01)	No effect
L-Tryptophan	0.09	7.37 (0.08)	34% Activation
	0.18	8.31 (0.06)	51% Activation
	0.35	11.28 (0.10)	105% Activation
	0.70	3.36 (0.02)	39% Inhibition
	3.50	0.94 (0.02)	83% Inhibition
L-Ascorbic acid	0.7	5.50 (0.01)	No effect
	7.0	3.41 (0.03)	38% Inhibition
	70.0	2.48 (0.03)	55% Inhibition
L-Malic acid	0.7	5.50 (0.00)	No effect
	7.0	5.50 (0.02)	No effect
	70.0	4.73 (0.07)	14% Inhibition
Citric acid	0.7	5.50 (0.01)	No effect
	7.0	5.50 (0.01)	No effect
	70.0	2.81 (0.03)	49% Inhibition

Figure IV-1. Changes in the anthocyanin content of control and mannitol- stressed cells at different days after culture inoculation. Cells were subjected to osmotic stress with mannitol by inoculating the culture in the medium of -1.6 MPa osmotic potential, obtained by the addition of mannitol to the MS medium containing 105 mM glucose (medium osmotic potential of -0.6 MPa), which served as the control. Each point is the mean of three replications. Error bars represent +/- standard error of the mean (SEM). F test for osmotic stress treatment, harvest day and their interaction were significant at P-0.01. Least significant difference (LSD, $P<0.05$) for mean comparison = 0.43.



Control (-0.6 MPa)
Mannitol -1.6 MPa
Anthocyanin [(µmol) (g dry weight)$^{-1}$]
0
1
2
3
4
5
0
3
6
9
12
Harvest day

Figure IV-2. Specific activities of phenylalanine ammonia lyase (PAL) (A) and tyrosine ammonia lyase (TAL) (B), and the ratio of PAL activity to TAL activity (C) of control and mannitol stressed cells at different days after culture inoculation. Treatments were imposed as described in Figure IV-1. Each point/bar is the mean of three replicates. Error bars represent SEM. F test for osmotic stress treatment, and interaction of treatment and harvest day was significant at $P<0.01$, for both PAL and TAL activities and their ratio. LSD ($P<0.05$) values for mean comparison for PAL activity = 0.35, for TAL activity = 0.03 and for the ratio of PAL activity to TAL activity = 2.35.

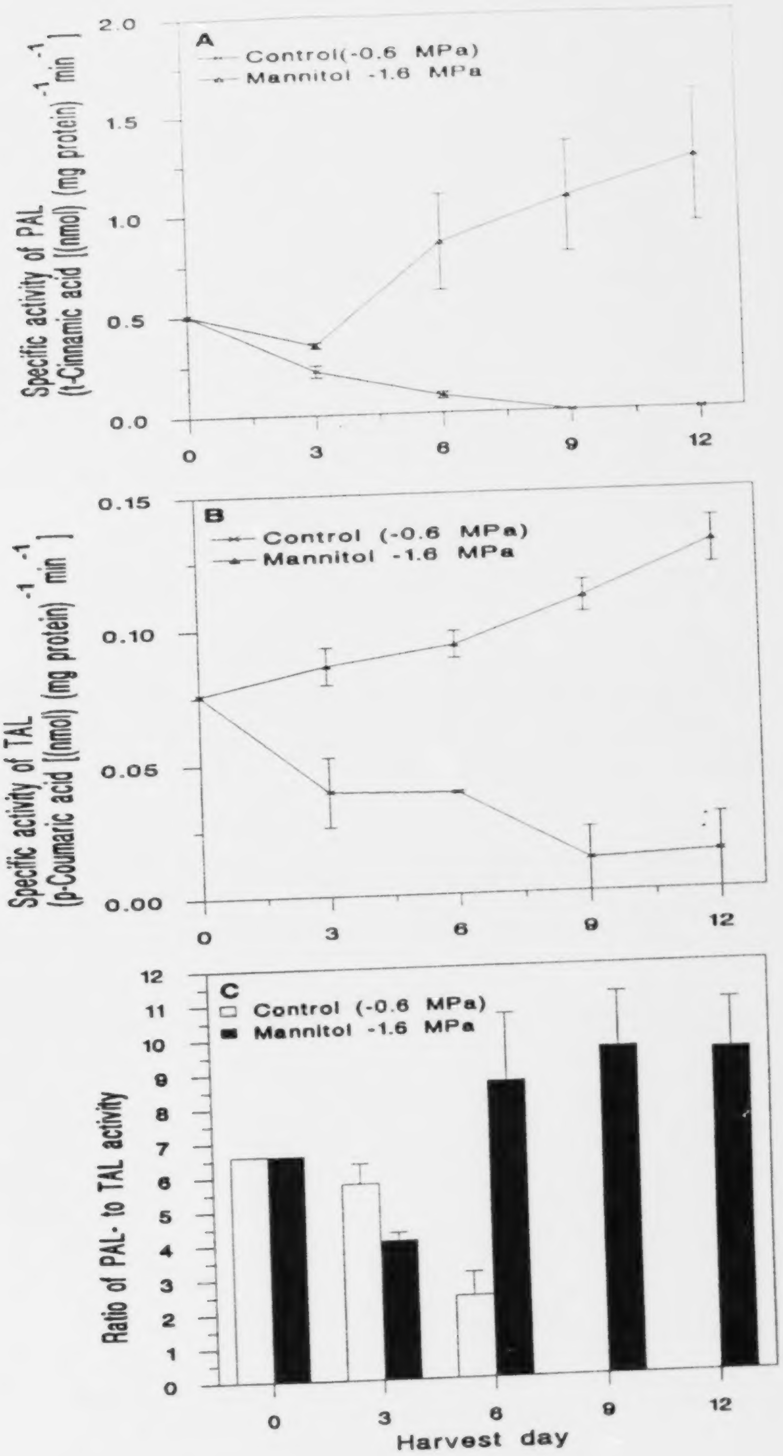

A
Control(-0.6 MPa)
Mannitol -1.6 MPa
Specific activity of PAL
(t-Cinnamic acid [(nmol) (mg protein)$^{-1}$ min^{-1}]
2.0
1.5
1.0
0.5
0.0
0
3
6
9
12
B
Control (-0.6 MPa)
Mannitol -1.6 MPa
Specific activity of TAL
(p-Coumaric acid [(nmol) (mg protein)$^{-1}$ min^{-1}]
0.15
0.10
0.05
0.00
0
3
6
9
12
C
Control (-0.6 MPa)
Mannitol -1.6 MPa
Ratio of PAL- to TAL activity
12
11
10
9
8
7
6
5
4
3
2
1
0
0
3
6
9
12
Harvest day

Figure IV-3. PAL and TAL activities per unit volume of eluent from sephadex G-200 chromatography of partially purified enzyme extract of cells maintained in MS medium. Three to five ml fractions were collected and both PAL and TAL activities were determined in each fraction containing protein (absorbance @ 280 nm).

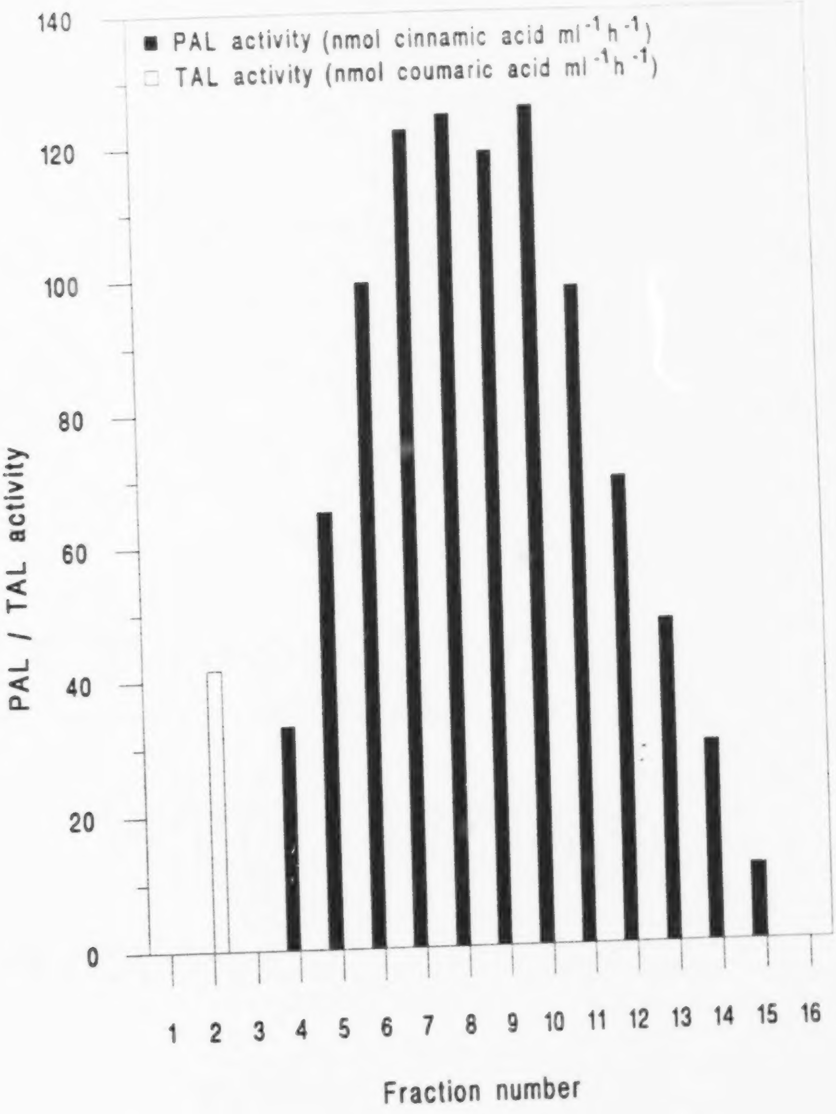

■ PAL activity (nmol cinnamic acid $ml^{-1} h^{-1}$)
☐ TAL activity (nmol coumaric acid $ml^{-1} h^{-1}$)
PAL / TAL activity
0
20
40
60
80
100
120
140
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Fraction number

Figure IV-4. Changes in protein content of control and mannitol stressed cells at different days after culture inoculation. Treatments were imposed as described in Figure IV-1. Each point is the mean of three replicates. Error bars represent +/- SEM. F test for osmotic stress treatment and interaction of osmotic stress and harvest day was significant at $P<0.01$. LSD ($P<0.05$) for mean comparison = 13.0.

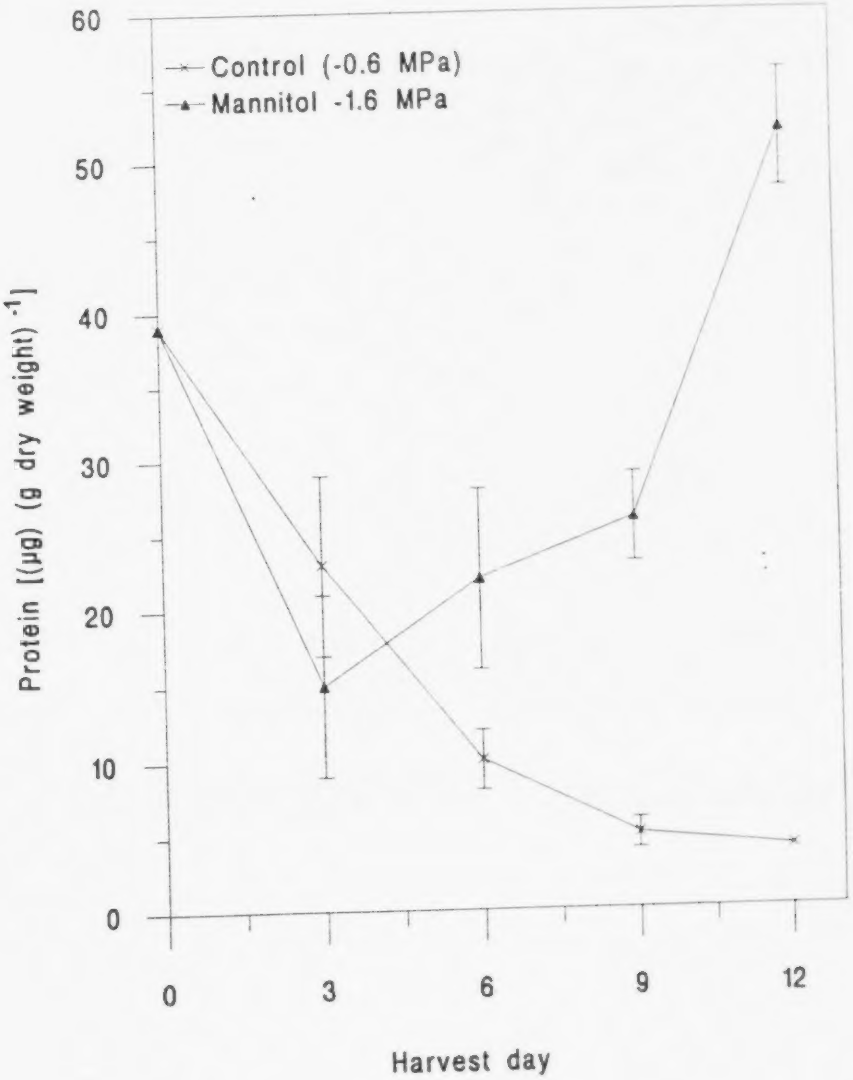
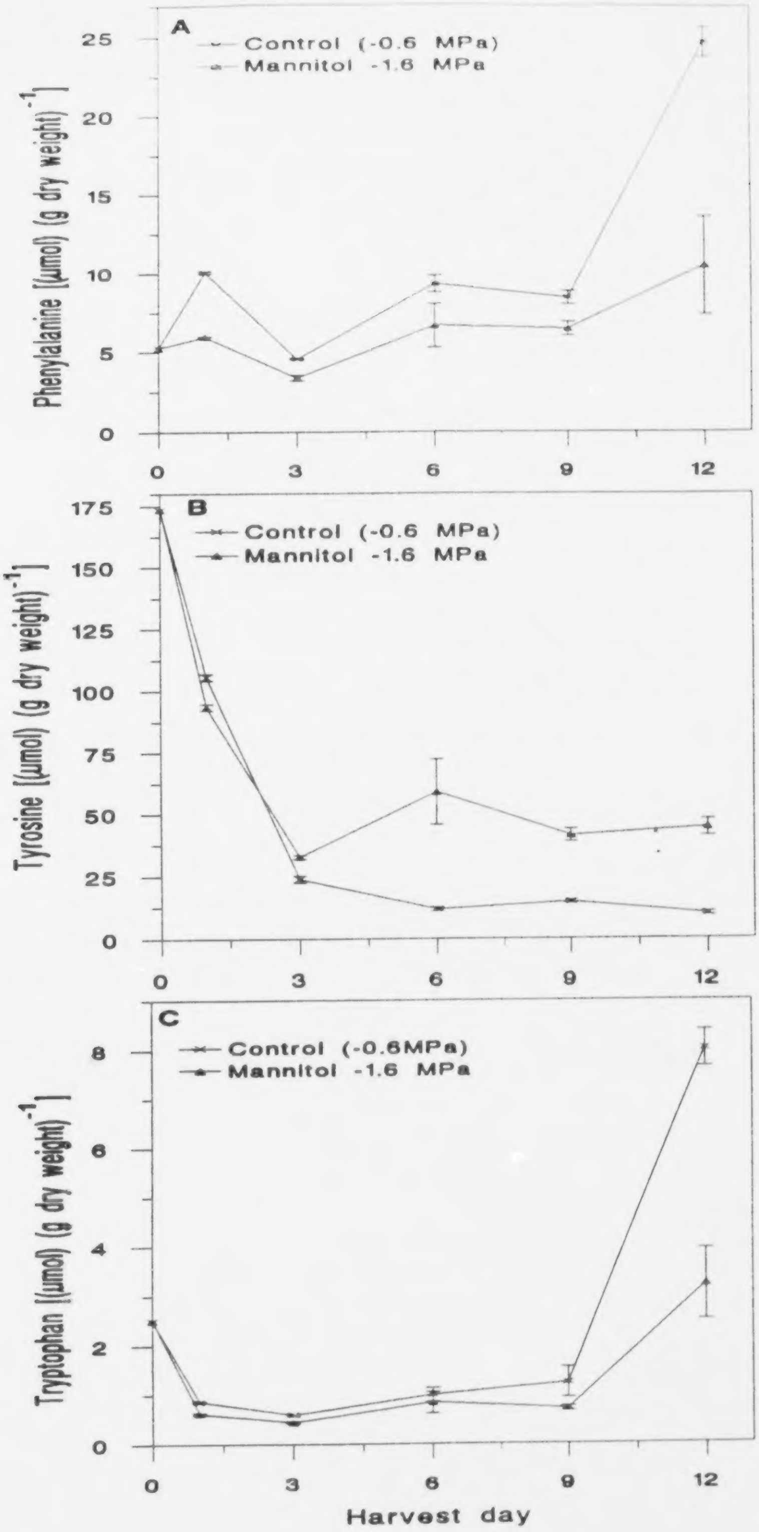

60
50
40
30
20
10
0
Protein [(µg) (g dry weight) -1]
Control (-0.6 MPa)
Mannitol -1.6 MPa
0
3
6
9
12
Harvest day

Figure IV-5. Changes in phenylalanine (A), tyrosine (B) and tryptophan (C) content of control and mannitol stressed cells at different days after culture inoculation. Treatments were imposed as described in Figure IV-1. Each point is the mean of three replicates. Error bars represent +/- SEM. F test for osmotic stress treatment, harvest day and their interaction was significant at $P<0.01$ for phenylalanine, tyrosine and tryptophan. LSD ($P<0.05$) for mean comparison for phenylalanine = 3.41, for tyrosine = 13.29 and for tryptophan = 0.84.


A
Control (-0.6 MPa)
Mannitol -1.6 MPa
Phenylalanine [(µmol) (g dry weight)$^{-1}$]
0
5
10
15
20
25
0
3
6
9
12
B
Control (-0.6 MPa)
Mannitol -1.6 MPa
Tyrosine [(µmol) (g dry weight)$^{-1}$]
0
25
50
75
100
125
150
175
0
3
6
9
12
C
Control (-0.6MPa)
Mannitol -1.6 MPa
Tryptophan [(µmol) (g dry weight)$^{-1}$]
0
2
4
6
8
0
3
6
9
12
Harvest day

IV.5 References

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Chapter V

GENERAL DISCUSSION AND CONCLUSION

Investigations were conducted using a cell suspension culture of poplar (*Populus deltoides* var. *occidentalis)*, which accumulated the anthocyanin pigment cyanidin 3-glucoside in the lag phase of culture growth, to understand the role of solutes accumulated in response to osmotic stress on cell growth and anthocyanin production. Osmotic stress resulted in an increase in the anthocyanin content of the cells and caused a reduction in cell growth. A similar increase in secondary metabolite production due to osmotic stress and an inverse relationship between growth and secondary metabolism has been reported previously (Do & Cormier 1991a, 1991b & 1990; DiCosmo & Towers 1984). Based on the results of the present studies, the possible mechanisms by which an increase in anthocyanin content of the osmotically stressed cells may take place are presented schematically in Figure V-1. In the present study, a reduction in osmotic potential as well as water potential was observed in the osmotically stressed cells, which showed reduced growth and higher anthocyanin levels. Water stress is a well known environmental factor affecting plant growth (Gershenzon 1984). Although previous studies have shown that secondary metabolite production increases in cells grown in a low osmotic potential medium, there are no reports to show the correlation between the water potential or osmotic potential of the cells and secondary metabolite levels.

The reduction in the osmotic potential of stressed cells under investigation, occurred due to the increase in the organic solute content of the cells including the soluble carbohydrates, free amino acids and organic acids. Sugars, amino acids and organic acids are the major osmoregulatory solutes in

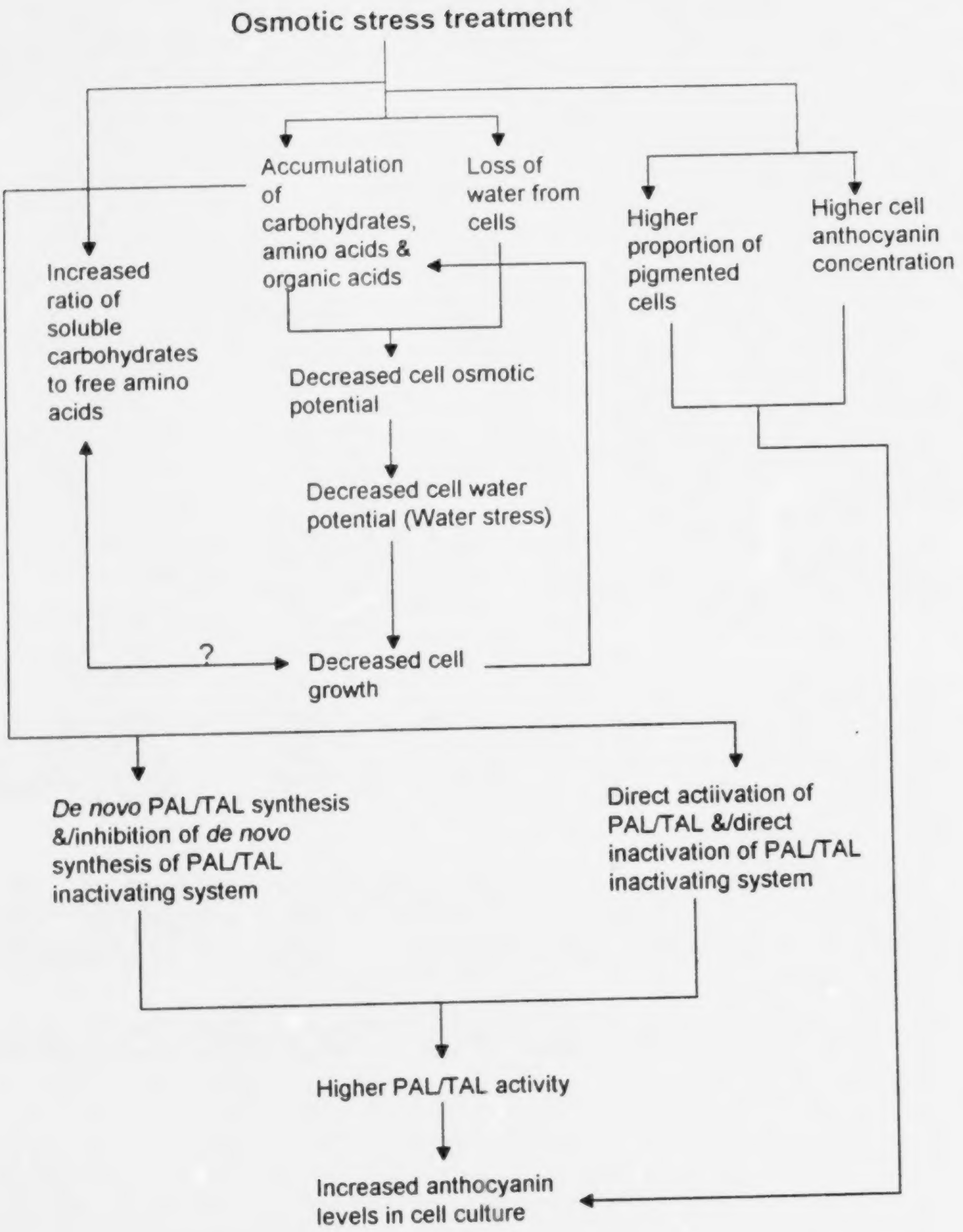
Osmotic stress treatment
Accumulation of carbohydrates, amino acids & organic acids
Loss of water from cells
Higher proportion of pigmented cells
Higher cell anthocyanin concentration
Increased ratio of soluble carbohydrates to free amino acids
Decreased cell osmotic potential
Decreased cell water potential (Water stress)
?
Decreased cell growth
De novo PAL/TAL synthesis &/inhibition of *de novo* synthesis of PAL/TAL inactivating system
Direct actiivation of PAL/TAL &/direct inactivation of PAL/TAL inactivating system
Higher PAL/TAL activity
Increased anthocyanin levels in cell culture

Figure V-1. Schematic presentation of the possible mechanisms causing an increase in the anthocyanin content of the osmotically stressed cells

many plant species (Fallon & Phillips 1988; Jacomini et al. 1988; Rudge & Morris 1987) including poplar (Tschaplinski & Blake 1989). This increase in the organic solute content of the osmotically stressed cells as found in this study, might have occurred due to the direct uptake of some of these solutes, hydrolysis of macromolecules and/or the accumulation of synthesized solutes due to growth inhibition.

In the present study, an increase in the anthocyanin content of the cells occurred with the increase in the level of osmotic stress. I have studied the effect of not only the quantitative aspect of osmotic stress but also the qualitative aspect of the osmotic solute on growth and anthocyanin production. The qualitative aspect of osmotic solute on growth and anthocyanin accumulation became apparent from studies comparing mannitol and glucose induced osmotic stress (Chapter III). My studies showed that at the same initial osmotic potential of the medium, mannitol stress was less effective in inhibiting cell growth and stimulating anthocyanin production than glucose stress. Accumulation of mannitol, a compatible solute (Borowitzka 1981), in mannitol stressed cells might have been the cause for this difference. Although previous studies have shown the quantitative effect of osmotic stress on secondary metabolite production, the qualitative effect of the osmotic solute on cell growth and secondary metabolite production has not been studied.

The other qualitative aspect of the osmotic solute, the ratio of soluble sugars to amino acids was found to have an effect on growth and anthocyanin production in this cell culture. This was apparent by a comparison of the osmotic stress induced by mannitol and glucose, which showed that higher ratio of soluble sugars to amino acids in the cell caused greater growth inhibition and higher anthocyanin accumulation. The ratio of soluble sugar to amino acid content of the cells was better correlated with the anthocyanin content of the cells compared to either the total soluble sugar or the total free amino acid content.

I found that both an increase in the proportion of anthocyanin producing cells and an increase in the concentration of anthocyanin in the productive cells was responsible for the increase in the anthocyanin content of a unit weight or volume of osmotically stressed cells. Even at the maximum anthocyanin accumulating stage, a large proportion of cells were unproductive and a large variation in the anthocyanin content among the productive cells occurred, indicating that a potential for further increases in anthocyanin content of the cell culture existed. Cellular heterogeneity appears to be a common feature of plant cell cultures. Such large variation in the anthocyanin content among the productive cells and preponderance of nonproductive cells has been observed in cell cultures of grapes (Do & Cormier 1990) and *Catharanthus roseus* (Hall & Yeoman 1986). The presence of cell aggregates, as observed in this cell culture, might be one of the reasons for this variation in the anthocyanin content of the cells. The use of fine cell suspension cultures containing small cell aggregates might be a possible means of improving the yields of secondary metabolites in cell cultures.

I found both phenylalanine ammonia lyase (PAL, EC 4.3.1.5) and tyrosine ammonia lyase (TAL, EC 4.3.1) activities in the poplar cells in suspension culture. TAL was found to be a different enzyme and PAL did not show TAL activity. An increase in the activities of both the enzymes occurred in osmotically stressed cells (Chapter IV), suggesting a regulatory role for both the enzymes in anthocyanin biosynthesis of osmotically stressed cells. PAL is considered as a regulatory enzyme of secondary metabolism (Hanson & Havir 1981). While PAL has been detected in almost all the plant species examined, TAL activity has been detected mainly in monocotyledons, and in a few species of dicotyledons such as *Catharanthus* (Nagaoka & Ashihara 1988), tobacco (Beaudoin-Eagan & Thorpe 1985) and castor bean (Gregor 1976). The present study appears to be the first report showing the presence of TAL in poplar and the first report showing a correlation between TAL activity and anthocyanin production. PAL activity was however, higher than TAL activity and therefore, PAL might be more important than TAL in the regulation of anthocyanin biosynthesis in this cell culture. In almost all the plant species examined so far, PAL activity was higher than TAL activity (Hanson & Havir 1981; Nagaoka & Ashihara 1988).

The solutes accumulated in osmotically stressed cells might have influenced PAL activity either by its direct activation or by direct inhibition of the PAL inactivating system. My findings showed that the aromatic amino acids tyrosine and tryptophan increased *in vitro* PAL activity. Hence both of them may have a role in increasing the activity of the existing pool of PAL *in vivo*. Behal (1985) observed that PAL activity was increased in the basidiomycete *Oudemansiella mucida* by tryptophan supplied through the medium. However, Koukol & Conn (1961) found L-tyrosine to be inhibitory to *in vitro* activity of PAL from barley.

Determination of PAL protein content by ELISA showed that higher amount of PAL protein was present in osmotically stressed poplar cells. Therefore, increased PAL activity of osmotically stressed cells might also have resulted from its *de novo* synthesis or inhibition of *de novo* synthesis of PAL inactivating system, brought about by accumulated solutes. Various amino acids, when supplied through the medium have been shown to increase PAL activity in the yeast *Rhodotorula glutinis* by increasing its *de novo* synthesis. Isoleucine was found to contribute to the stability of PAL activity by inhibiting the synthesis of the PAL inactivating system (Nakamichi et al. 1983). *De novo* synthesis of PAL contributing to increased PAL activity and the involvement of a PAL inactivating system in the regulation of PAL activity has been discussed by Jones (1984) and Hanson & Havir (1981).

Investigations on the production of secondary metabolites should be aimed at determining whether substrate supply or enzyme activity is the limiting factor for their synthesis. By a comparing the changes in cellular phenylalanine content and PAL activity, it appears that the cellular content of the substrate of PAL, phenylalanine, might not have influenced PAL activity, and hence was probably not responsible for increased anthocyanin production in osmotically stressed cells. These results are contrary to the previous findings that PAL activity is regulated by phenylalanine supply (Cunha 1987). Increased anthocyanin production in the osmotically stressed cells under study was associated with a reduction in cell growth. However, the findings of the present investigations that phenylalanine supply was not the limiting factor for PAL activity and that the protein content of the cells was positively correlated with PAL activity as well as anthocyanin content, emphasizes the need for a better understanding of the relationship between primary and secondary metabolism.

Because osmotic stress was found to result in an increase in the cellular soluble sugar content, especially of glucose, it would be interesting to find out if glycosylation of the anthocyanidin is influenced by an increase in the glucose content of the osmotically stressed cells. Also, it should be determined if the post-translational modification of PAL, in which a carbohydrate which is supposed to be associated with PAL (Havir 1979) might be attached to the PAL molecule, is influenced by osmotic stress. The possible role of the higher amino acid content of the stressed cells in the formation of the dehydroalanine containing prosthetic group of PAL (Hanson & Havir 1981) also needs to be determined.

In addition to the organic solutes, the effect of other solutes, such as ions accumulated in response to osmotic stress, on enzyme activity and the anthocyanin accumulation process needs further investigation. The exact signal for the increased *de novo* synthesis of PAL induced by osmotic stress either at the transcription or at the translation level needs to be understood. A better understanding of the mechanism of regulation of secondary metabolite production might provide methods to enhance their synthesis.

It might be possible to extend the findings of this study to enhance the yields of secondary metabolites of high economic value. By a better understanding of the cause for the heterogeneity of a particular cell culture system, it might be possible to induce secondary metabolite accumulation in a larger proportion of cells, thereby increasing the yield of a secondary metabolite. Determination of the regulatory enzymes in the biosynthesis of a secondary metabolite for a particular culture system and determination of the methods to induce their *de novo* synthesis as well as activation, might be helpful in inducing higher enzyme activity and higher product synthesis. Modifying the cells by genetic engineering techniques to induce high synthesis of the key enzymes might be a useful approach towards increasing the yields of secondary metabolites in cell cultures. This might make the production of certain secondary metabolites of high economic value commercially feasible.

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Appendix 1. Significance level of F test for osmotic stress treatment, harvest day and their interactions for the various free amino acids present in the cells at different days after culture initiation.

Amino acid	Source of variation		
	Treatment (T)	Harvest day (H)	T x H
Alanine	0.01	0.01	0.01
Aspartic acid	0.05	0.01	NS
Isoleucine	0.01	0.01	0.01
Leucine	0.01	0.01	0.01
Glycine	NS	0.01	NS
Methionine	0.01	0.01	0.01
Valine	NS	0.01	0.01
Glutamine	0.01	0.01	0.01
Glutamic acid	0.05	0.01	0.01
Aspargine	0.01	0.01	0.01
Serine	0.01	0.01	0.05
Histidine	0.01	0.01	0.01
Lysine	0.05	0.01	0.01
Threonine	NS	0.01	0.01
Ornithine	NS	0.01	NS
Arginine	NS	0.01	0.01
Total	0.01	0.01	0.01

Note: 0.01= Significant at P<0.01
0.05= Significant at P<0.05
NS = Nonsignificant

Appendix 2 Significance level of F test for osmotic stress treatment, harvest day and their interactions for the various organic acids present in the cells at different days after culture initiation.

Organic acid	Source of variation		
	Treatment (T)	Harvest day (H)	T x H
Shikimic acid	NS	NS	0.05
Ascorbic acid	0.01	0.05	NS
Citric acid	0.05	0.01	NS
Total	0.01	0.01	0.05

Note: 0.01= Significant at $P<0.01$

0.05= Significant at $P<0.05$

NS = Nonsignificant

Appendix 3. Significance level of F test for osmotic stress treatment, harvest day and their interactions for the various soluble carbohydrates present in the cells at different days after culture initiation.

Soluble carbohydrate	Source of variation		
	Treatment (T)	Harvest day (H)	T x H
Sucrose	0.01	0.01	0.01
Glucose	0.01	0.01	0.05
Fructose	0.01	0.01	0.01
Inositol	0.01	0.01	0.01
Mannitol	0.01	0.01	0.01
Total	0.01	0.01	0.01

Note: 0.01= Significant at $P<0.01$
0.05= Significant at $P<0.05$